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(54) Title: METHOD FOR CULTURING AND TRANSFORMING HUMAN STEM CELL-CONTAINING COMPOSITIONS

(57) Abstract

Methods, including culture media conditions and reactors, which provide for *ex vivo* human stem cell division and stable genetic transformation and/or the optimization of human hematopoietic progenitor cell cultures and/or increasing the metabolism or GM-CSF secretion or IL-6 secretion of human stromal cells are disclosed. The methods rely on culturing human stem cells and/or human hemapoietic progenitor cells and/or human stromal cells in a liquid culture medium which is replaced, preferably perfused, either continuously or periodically, at a rate of .1 ml of medium per ml of culture per about 24 to about 48 hour period, and removing metabolic products and replenishing depleted nutrients while maintaining the culture under physiologically acceptable conditions. Optionally growth factors are added to the culture medium.

+ DESIGNATIONS OF "SU"

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Description

METHOD FOR CULTURING AND TRANSFORMING HUMAN STEM CELL-CONTAINING COMPOSITIONS

5

Technical Field

10   This invention relates to methods, reactors and compositions for the growth and transformation of mammalian cells in culture, particularly the growth and transformation of hematopoietic cell cultures.

Background Art

15   All of the circulating blood cells in the normal adult, including erythrocytes, leukocytes, platelets and lymphocytes, originate as precursor cells within the bone marrow. These cells, in turn, derive from very immature cells, called progenitors, which are assayed by their

20   development into contiguous colonies of mature blood cells in 1-3 week cultures in semisolid media such as methylcellulose or agar. Progenitor cells themselves derive from a class of progenitor cells called stem cells. Stem cells have the capacity, upon division, for both self-

25   renewal and differentiation into progenitors. Thus, dividing stem cells generate both additional primitive stem cells and somewhat more differentiated progenitor cells. In addition to the generation of blood cells, stem cells also may give rise to osteoblasts and osteoclasts, and

30   perhaps cells of other tissues as well. This document describes methods and compositions which permit, for the first time, the successful *in vitro* culture of human hematopoietic stem cells, which results in their proliferation and differentiation into progenitor cells and

-2-

more mature blood cells.

In the late 1970s the liquid culture system was
developed for growing hematopoietic bone marrow *in vitro*.
The cultures are of great potential value both for the
5   analysis of normal and leukemic hematopoiesis and for the
experimental manipulation of bone marrow, for, e.g.,
retroviral-mediated gene transfer. These cultures have
allowed a detailed analysis of murine hematopoiesis and
have resulted in a detailed understanding of the murine
10   system. In addition, it has made possible retroviral gene
transfer into cultured mouse bone marrow cells. This
allowed tagging murine hematopoietic cells proving the
existence of the multi-potent stem cell and of the study of
the various genes in the process of leukemogenesis.

15   But while it has been possible to transfer retroviral
genes into cultured mouse bone marrow cells, this is not
yet been possible in cultured human bone marrow cells
because, to date, human long-term bone marrow cultures have
been limited both in their longevity and in their ability
20   to maintain stem cell survival and their ability to produce
progenitor cells over time.

Human liquid bone marrow cultures were initially found
to have a limited hematopoietic potential, producing
decreasing numbers of progenitor cells and mature blood
25   cells, with cell production ceasing by 6 to 8 weeks.
Subsequent modifications of the original system resulted
only in modest improvements. A solution to this problem is
of incalculable value in that it would permit, e.g.,
expanding human stem cells and progenitor cells for bone
30   marrow transplantation and for protection from
chemotherapy, selecting and manipulating such cells, i.e.,
for gene transfer, and producing mature human blood cells

-3-

for transfusion therapy.

Studies of hematopoiesis and *in vitro* liquid marrow
cultures have identified fibroblasts and endothelial cells
within adhering layers as central cellular stromal
5   elements. These cells both provide sites of attachment for
developing hematopoietic cells and can be induced to
secrete hematopoietic growth factors which stimulate
progenitor cell proliferation and differentiation. These
hematopoietic growth factors include granulocyte colony
10   stimulating factor (G-CSF), granulocyte-macrophage colony
stimulating factor (GM-CSF) and interleukin-6 (IL-6).

Cultures of human bone marrow cells on such adherent
layers *in vitro* however have been largely disappointing.
Unlike related cultures from other species, such as mouse
15   and tree shrew, human liquid marrow cultures fail to
produce significant numbers of either nonadherent
hematopoietic precursor cells or clonogenic progenitor
cells for over 6 to 8 weeks. And although cultures lasting
3-5 months have been reported, no culture which stably
20   produces progenitor cells from stem cells continuously for
more than 4-6 weeks has been reported.

Moreover, nonadherent and progenitor cell production
typically declined throughout even the short life of these
cultures, so that it is not clear that stem cell survival
25   or proliferation is supported at all by these cultures.
Further, when studied in isolation, unstimulated bone
marrow stromal cells secrete little if any detectable
hematopoietic growth factors (HGFs).

The lack of stable progenitor cell and mature blood
30   cell production in these cultures has led to the belief
that they are unable to support continual stem cell renewal

and expansion. It has therefore been presumed that the cultures either lack a critical stem cell stimulant(s) and/or contain a novel stem cell inhibitor(s). But while explanations for failure to detect HGFs and uninduced

5 stromal cell cultures have been suggested, the null hypothesis, which combines the failure to detect HGFs and the relative failure of human liquid marrow cultures, would be that the culture systems used *in vitro* do not provide the full range of hematopoietic supportive function of

10 adherent bone marrow stromal cells *in vivo*.

Stem cell and progenitor cell expansion for bone marrow transplantation is a potential application for human long-term bone marrow cultures. Human autologous and allogenic bone marrow transplantation are currently used as

15 therapies for diseases such as leukemia, lymphoma and other life-threatening disorders. For these procedures however, a large amount of donor bone marrow must be removed to insure that there is enough cells for engraftment.

A culture providing stem cell and progenitor cell

20 expansion would reduce the need for large bone marrow donation and would make possible obtaining a small marrow donation and then expanding the number of stem cells and progenitor cells *in vitro* before infusion into the recipient. Also, it is known that a small number of stem

25 cells and progenitor cells circulate in the blood stream. If these stem cells and progenitor cells could be collected by phoresis and expanded, then it would be possible to obtain the required number of stem cells and progenitor cells for transplantation from peripheral blood and

30 eliminate the need for bone marrow donation.

Bone marrow transplantation requires that approximately 1×10^8 to 2×10^8 bone marrow mononuclear

-5-

cells per kilogram of patient weight be infused for
engraftment. This requires the bone marrow donation of the
same number of cells which is on the order of 70 ml of
marrow for a 70 kg donor. While 70 ml is a small fraction
5   of the donors marrow, it requires an intensive donation and
significant loss of blood in the donation process. If stem
cells and progenitor cells could be expanded ten-fold, the
donation procedure would be greatly reduced and possibly
involve only collection of stem cells and progenitor cells
10   from peripheral blood and expansion of these stem cells and
progenitor cells.

     Progenitor cell expansion would also be useful as a
supplemental treatment to chemotherapy, and is another
application for human long-term bone marrow cultures. The
15   dilemma faced by oncologist is that most chemotherapy
agents used to destroy cancer act by killing all cells
going through cell division. Bone marrow is one of the
most prolific tissues in the body and is therefore often
the organ that is initially damaged by chemotherapy drugs.
20   The result is that blood cell production is rapidly
destroyed during chemotherapy treatment and chemotherapy
must be terminated to allow the hematopoietic system to
replenish the blood cell supply before a patient is
retreated with chemotherapy. It may take a month or more
25   for the once quiescent stem cells to raise up the white
blood cell count to acceptable levels to resume
chemotherapy during which case the drop in blood cell count
is repeated. Unfortunately, while blood cells are
regenerating between chemotherapy treatments, the cancer
30   has time to grow and possibly become more resistant to the
chemotherapy drugs due to natural selection.

     To shorten the time between chemotherapy treatments,
large numbers of progenitor and immature blood cells could

be given back to the patient. This would have the effect
of greatly reducing the time the patient would have low
blood cell counts, thereby allowing more rapid resumption
of the chemotherapy treatment. The longer chemotherapy is
5   given and the shorter the duration between treatments, the
greater the odds of successfully killing the cancer.

The hematopoietic cells required for progenitor cell
expansion may come from either bone marrow withdrawal or
peripheral blood collection. Bone marrow harvests would
10 result in collection of approximately 4 x 10^5 CFU-GM
progenitor cells. Phoresis of 5 liters of peripheral blood
would collect approximately 10^5 CFU-GM although this number
could be increased to 10^6 CFU-GM by prior treatment of the
donor with GM-CSF. Rapid recovery of a patient would
15 require transfusion of approximately 1 x 10^8 to 5 x 10^8 CFU-
GM which is 100 to 1,000 times more than obtained by
routine bone marrow donation or by peripheral blood
donation. Therefore, expansion of bone marrow or
peripheral blood to increase the number of CFU-GM 2 to 3
20 orders of magnitude would significantly affect chemotherapy
administration and cancer treatment.

Gene therapy is a rapidly growing field in medicine
which is also of inestimable clinical potential. Gene
therapy has many potential uses in treating disease and has
25 been reviewed extensively. See, e.g., Boggs, Int. J. Cell
Cloning. (1990) 8:80-96, Kohn et al, Cancer Invest. (1989)
7 (2):179-192, Lehn, Bone Marrow Transp. (1990) 5:287-293,
and Verma, Scientific Amer. (1990) pp. 68-84. Genetically
transformed human stem cells have wide potential
30 application in clinical medicine, as agents of gene
therapy.

Gene therapy describes an emerging approach to

-7-

clinical treatment which has evolved from earlier
approaches in medical care. The earliest approaches to
medical care, evolving over centuries, included gross
surgical procedures and the administration of crude

5   mixtures as medicinal agents. In the past century,
biochemical pharmacology has supervened as the major method
of medical treatment. Under this paradigm, pure
biochemical molecules are delivered to the patient. In
general, such pharmacologic agents act either as poisons

10   (such as antimicrobials or cancer chemotherapy agents),
physiologic mimetics which stimulate endogenous receptors
(e.g., opiates, adrenergic agonists), or physiologic
antagonists which block endogenous receptors (e.g.
antihypertensives, anaesthetics).

15   Gene therapy is, by definition, the insertion of genes
into cells for the purpose of medicinal therapy. The
principle underlying gene therapy is to, rather than
deliver doses of pharmacologic molecules, deliver a
functional gene whose RNA or protein product will produce

20   the desired biochemical effect in the target cell or
tissue. There are several potential advantages of gene
therapy over classical biochemical pharmacology. First,
inserted genes can produce extremely complex molecules,
including RNA and protein, which can be extraordinarily

25   difficult or impossible to administer and deliver
themselves. Next, controlled insertion of the desired gene
into specific target cells can control the production of
gene product to defined tissues. Finally, gene therapy can
in principle be permanent within an individual, as the gene

30   will continue to function in the target cells and their
progeny.

         There are several problems that must therefore be
addressed for successful gene therapy. The first is to be

able to insert the desired therapeutic gene into the chosen
cells. Second, the gene must be adequately expressed in
the target cell, resulting in the appropriate levels of
gene product. Finally the RNA or protein produced must be
5 properly processed by the target cell so that it is
functional, i.e. so that gene therapy actually infers
clinical therapy. Several methods of gene insertion into
human cells *in vitro* are listed in Table 1.

Table 1:   Comparison of DNA transfer methods.

Variable	Microinjection	Electroporation	Retrovirus
Efficiency	10-100%	0.0001-1%	1-100% (depends on titer)
Effort	High	Low	Intermediate
Expense	High	Low	Intermediate
Stability	Good	Good	May be inactivated or become infective
DNA synthesis	?	?	Required
Size of DNA input	Not restricted	Not restricted	Limited (≤8 kb)
Need extraneous DNA	No	No	Yes

Other techniques, such as homologous recombination,
are being developed as well in many laboratories. Research
20 in gene therapy has been on-going for several years in
several types of cells *in vitro*, progressed to animal
studies, and has recently entered the first human clinical
trial(s).

The hematopoietic system is an ideal choice as a
25 delivery system for gene therapy. Hematopoietic cells are
readily accessible, simply by bone marrow aspiration or by
peripheral blood mononuclear cell harvest. Once the
genetic insertion is accomplished *in vitro* the treated
cells can be reinfused intravenously, after which the
30 genetically transformed cells will home to and develop in
the bone marrow. Since mature blood cells circulate

-9-

throughout the body, the genetically modified cells can
deliver the specific gene product to any desired tissue.

Most importantly, hematopoietic tissues contain stem
cells, which possess extensive (perhaps unlimited)
5   capacities for self-renewal. This implies that if genetic
material were stably transduced into these stem cells, then
upon reinfusion of the hematopoietic tissue, these altered
stem cells can expand and repopulate the marrow with cells
that express the new gene. This leads to long-lasting,
10   perhaps lifelong delivery of the desired gene product.
Similarly, successful stable gene transfer into stem cells
located in other tissues, or into embryonic stem cells,
likewise leads to long-lasting gene product delivery.

Successful hematopoietic stem cell gene therapy has
15   broad application, to both diseases specific to the
hematopoietic system and to other organ system diseases.
Within the hematopoietic system, both inherited and
acquired diseases can be treated by stem cell gene therapy.
For example, hemoglobin deficiencies such as α and β
20   Thalessemias could be treated by the insertion of the gene
coding for the globin α or β chain, together with
regulatory sequences that confer high level tissue specific
erythrocytes (see, Grosveld et al, Cell (1987) 51:975-986).
Similarly, sickle cell anemia could be corrected by the
25   genetic insertion of the fetal globin gene into
hematopoietic stem cells, as the regulated expression of
high levels of fetal hemoglobin are sufficient to prevent
sickling in red cells despite the copresence of sickle
hemoglobin (see, Sunshine et al, J. Molec. Biol. (1979)
30   133:435).

Genetic disease of neutrophils caused by functional
protein deficiencies, such as leukocyte adhesion deficiency

(LAD) or chronic granulomatous disease (CGD) could be
treated by the genetic insertion of the gene encoding the
defective or absent gene, along with regulatory DNA
sequences that confer high level, tissue specific

5   expression into hematopoietic stem cells (see, Wilson et
al, Science (1990) 248:1413-1416). Genetic diseases
involving platelets, such as von Willebrands' Disease,
could be corrected by the genetic insertion of the gene
encoding, e.g. von Willebrands' Factor, along with

10   sequences which permit its expression and secretion.

      The particular suitability of hematopoietic stem cell
gene therapy for the replacement of congenitally deficient
gene products is particularly evident in the treatment of
lymphocyte immunodeficiency diseases, such as severe

15   combined immunodeficiency due to adenosine deaminase
deficiency. Retroviral gene therapy of circulating T cells
with the ADA gene has been found to be successful at
reducing the clinical immunodeficiency experienced by these
patients, but the effects are only temporary because the

20   transfected T lymphocytes have a finite life span in vivo
(see, Kasid et al, Proc. Nat. Acad. Sci. (USA) (1990)
87:473-477, or Culver et al Proc. Nat. Acad. Sci. (USA),
(1991) 88:3155-3159). If, however, the gene could be
successfully transfected into hematopoietic stem cells,

25   then all of the T cells which arose from these stem cells
would contain and express the ADA gene. Therefore, since
the transfected stem cells would persist and proliferate
for the life of the patient, the T cell ADA deficiency
would be permanently treated by a single gene transfer stem

30   cell treatment (see, Wilson et al, Proc. Natl. Acad. Sci.,
(U.S.A.) (1990) 87:439-443).

      In addition to treating inherited enzymatic
abnormalities of the hematopoietic system, stem cell gene

-11-

therapy could be useful for protecting stem cells and their
progeny from toxic exogenous agents such as viruses or
chemotherapy. For example, gene transfer of DNA sequences
encoding the TAR binding site of the HIV TAT

5   transactivating factor have been shown to protect T cells
from spreading infection by the HIV virus (see, Sullenger
et al, Cell (1990) 63:601-608). Stable transaction of
these sequences into hematopoietic stem cells would result
in a pool of T cells, all arising from these stem cells,

10 which were relatively or absolutely resistant to the spread
of HIV.

    Similarly, successful transfection of the genes
encoding the multi-drug resistance gene (MDR) or the
methotrexate resistance gene into human bone marrow stem

15 cells would create stem cells which were relatively
resistant to the effects of cancer chemotherapy. Following
autologous bone marrow transplantation with these
genetically manipulated cells, patients would be able to
tolerate chemotherapy with the agents to which their stem

20 cells were protected with suffering the profound bone
marrow suppression commonly caused by these anti-cancer
drugs. This would enable patients to receive more effect
doses of cancer chemotherapy with less toxicity.

    One can readily envision that hematopoietic stem cell

25 gene therapy will also be useful for acquired hematopoietic
disease such as leukemia, lymphoma and aplastic anemia.
Once the genetic causes of these diseases is discovered,
insertion of a gene whose product either overcomes that of
the abnormal gene in the cell or corrects it directly

30 (perhaps by splicing out and replacing the gene) would
correct the abnormality.

    On a broader level, however, hematopoietic stem cell

-12-

gene therapy can be useful for the treatment of diseases
outside the hematopoietic system as well. Gene transfer of
DNA sequences carrying therapeutic soluble proteins could
give rise to mature blood cells which permanently secreted
5   the desired amounts of a therapeutic molecule. By way of
examples, this approach could be useful for the treatment
of, e.g., diabetes mellitus by the insertion of DNA
sequences for insulin along with regulatory DNA sequences
that controlled the proper expression of the transfected
10 insulin gene, perhaps in response to elevated plasma
glucose levels. Systemic hypertension could be treated by
genetic insertion of stem cells with DNA sequences encoding
secretory peptides which act as competitive inhibitors to
angiotensin converting enzyme, to vascular smooth muscle
15 calcium channels, or to adrenergic receptors. Alzheimer's
disease could possibly be treated by genetic insertion into
stem cells of DNA sequences encoding enzymes which break
down amyloid plaques within the central nervous system.

The many applications of gene therapy, particularly
20 _via_ stem cell genetic insertion, are thus well known and
have been extensively reviewed (see, Boggs _et al_, _supra_,
Kohn _et al_, _supra_, Lehn, _supra_, and/or Verma _et al_, _supra_).
There are indeed increasing examples of some success in
achieving therapeutic gene transfer into differentiated
25 human stem cells, as described for example in T lymphocytes
(see, Kalsd _et al_, _Proc. Nat. Acad. Sci. (U.S.A.)_, (1990)
87:473-477, Culver _et al_, _Proc. Nat. Acad. Sci. (U.S.A.)_
(1991) 88:3155-3159).

Unfortunately, achieving (stable) gene transfer into
30 human stem cells has not been accomplished prior to the
present invention. While several groups have demonstrated
the feasibility of retroviral mediated gene transfer into
human hematopoietic cells, human primitive hematopoietic

-13-

stem cells have not been successfully transfected. This is
in sharp contrast to experiments in the mouse, in which
some level of retrovirally mediated gene transfer into
hematopoietic stem cells has been possible (see, <u>Wilson</u> <u>et</u>
5   <u>al</u>, <u>Pro. Nat. Acad. Sci. (USA)</u> (1990) <u>87</u>:439-443).

The major impediment to achieving successful human
hematopoietic stem cell gene therapy has been the inability
to insert genes into human hematopoietic cells under
conditions in which the stem cells are dividing and
10   proliferating. Successful stable gene insertion into a
target cell requires that the target cell undergo at least
one round of cell division. Thus if stem cells are not
dividing in the presence of the desired genetic material,
the material will not be stably inserted into the stem
15   cells. Prior to the development of the present invention,
no system existed which supported the *ex vivo* division and
proliferation of human stem and no successful genetic
transformation of human stem cells has been possible.

The literature which is relevant to the present
20   document also includes U.S. Patent No. 4,721,096 which
describes a 3-dimensional system involving stromal cells
for the growth of hematopoietic cells. See also references
cited therein. <u>Glanville et al</u>, <u>Nature</u> (1981) <u>292</u>:267-269,
describe the mouse metallothionein-I gene. <u>Wong et al</u>,
25   Science (1985) <u>228</u>:810-815, describe human GM-CSF.
<u>Lemischka et al</u>, <u>Cell</u> (1986) <u>45</u>:917-927, describe
retrovirus-mediated gene transfer as a marker for
hematopoietic stem cells and the tracking of the fate of
these cells after transplantation. <u>Yang et al</u>, <u>Cell</u> (1986)
30   <u>47</u>:3-10, describe human IL-3. <u>Chen et al</u>, <u>Mol. Cell. Biol.</u>
(1987) <u>7</u>:2745-2752, describe transformation of mammalian
cells by plasmid DNA. <u>Greaves et al</u>, <u>Cell</u> (1989) <u>56</u>:979-
986, describe the human CD2 gene. <u>Civin et al</u>, <u>J. Immunol.</u>

(1984) 133:1576, describe the CD34 antigen. <u>Martin et al</u>, <u>Cell</u> (1990) 63:203-211, describes human S-CSF; <u>Forrester et al</u>, <u>J Cell Science</u> (1984), 70:93-110, (1984), discuss parallel flow chamber. <u>Coulombel et al</u>, <u>J. Clin. Invest.</u>, 5 (1986) 75:961, describe the loss of CML cells in static cultures.

There is therefore a considerable need for methods and compositions for the *ex vivo* replication and stable genetic transformation of human stem cells and for the optimization 10 of human hematopoietic progenitor cell cultures, particularly in light of the great potential for stem cell expansion, progenitor cell expansion, and gene therapy offered by these systems. Unfortunately, to date, attempts to achieve such results have been disappointing.

15 <u>Disclosure of the Invention</u>
Accordingly, it is an object of this invention to provide novel methods, including culture media conditions and reactors, for the *ex vivo* replication and stable genetic transformation of human stem cells.

20 It is another object of this invention to provide novel methods, including culture media conditions and reactors, for the optimization (i) of human hematopoietic progenitor cell cultures and (ii) of obtaining stably genetically transformed human hematopoietic progenitor 25 cells.

The present invention is based on the inventors' discovery of novel methods, including culture media conditions and reactors, which provide for *ex vivo* human stem cell division and stable genetic transformation and/or 30 the optimization of human hematopoietic progenitor cell cultures. These methods rely on culturing human stem cells

-15-

and/or human hematopoietic progenitor cells in a liquid
culture medium which is replaced, preferably perfused,
either continuously or periodically, at a rate of 1
milliliter (ml) of medium per ml of culture per about 24 to
5   about 48 hour period, and removing metabolic products and
replenishing depleted nutrients while maintaining the
culture under physiologically acceptable conditions. In a
particularly preferred embodiment of the present invention,
the above medium replacement rate is used in conjunction
10   with the addition of hematopoietic growth factors to the
rapidly exchanged culture medium.

The inventors have discovered that the increased
medium exchange rate used in accordance with the present
invention, with the optional addition of hematopoietic
15   growth factors to the rapidly exchanged culture medium,
surprisingly (1) supports cultures in which human stem
cells proliferate over extended periods of time of at least
5 months, (2) supports cultures in which human
hematopoietic progenitor cells are produced by division and
20   differentiation of human stem cells through extended
culture periods of at least 5 months, and (3) stimulates
the increased metabolism of and GM-CSF secretion from human
stromal cells, including human bone marrow stromal cells.
The present invention provides, for the first time, human
25   stem cell survival and proliferation in culture.

The present invention also provides an *ex vivo* culture
system which supports the continuous proliferation of human
stem cells to allow the successful insertion of genetic
material into the human stem cells, resulting in the
30   creation of stably genetically transformed human stem
cells. This embodiment of the invention can be used for
the transfer of any genetic material that can be engineered
into a recombinant retrovirus, or any other gene transfer

-16-

vector that requires cell division. Genetically modified
human stem cells produced in this manner can be applied to
a wide variety of clinical diseases, as described supra.

5   The invention also provides methods for enhancing the
efficiency of genetic transfer into human hematopoietic
progenitor cells, together with providing stably
genetically transformed human stem cells and/or stably
genetically transformed human hematopoietic progenitor
cells.

10   Methods are also provided employing reactors and
compositions which allow for the efficient proliferation of
hematopoietic cells in culture, particularly cells at an
early stage in maturation, including stem cells. The
methods employ stromal cells, normally transformed, which
15   provide constitutive or inducible production of growth
factors, which cells are physically separated to allow for
easy separation of hematopoietic cells. By providing for
continuous perfusion, and recycling of cells as
appropriate, high densities and yields of viable
20   hematopoietic cells may be achieved. The reactor employs a
protein surface for the stromal cells and either the
surface or other barrier for maintaining separation of
stromal cells and hematopoietic cells.

Brief Description of the Figures
25   Fig. 1 is a schematic view of a perfusion
chamber;

Fig. 2 is a schematic representation and flow diagram
of the perfusion medium pathway;

Fig. 3a is a schematic view of a flow chamber for
30   measuring shear stress for separation of cells;

-17-

Fig. 3b is a side view of the flow chamber of Fig. 3a;

Fig. 3c is a graph of a shear stress profile for hematopoietic cells;

5   Figs. 4a and 4b are top and side views of a flow chamber for growing and separating hematopoietic cells; and

Figs 5a and 5b are views of a flow chamber in which barriers are removed sequentially allowing the continued growth of stromal cells.

Best Mode for Carrying Out the Invention

10   The advantages of the present invention may be observed whenever the present invention is applied to any standard system for liquid human stem and/or progenitor cell culture, such as those found in hematopoietic culture(s). By the use of the rapid medium exchange rates

15   used in accordance with the present invention, with the optional addition of supplementary hematopoietic growth factors to the culture, the inventors have surprisingly discovered that one is able to make standard systems for liquid human hematopoietic cultures, which comprise

20   cultures performed in the presence or absence of animal sera or plasmas, including horse, calf, fetal calf, or human serum, perform in a qualitatively superior manner.

Human liquid hematopoietic cultures which may be used in accordance with the invention can be performed at cell

25   densities of from 10^4 to 10^9 cells per ml of culture, using standard known medium components such as, for example, IMDM, MEM, DMEM, RPMI 1640, Alpha Medium or McCoy's Medium, which can use combinations of serum albumin, cholesterol and/or lecithin, selenium and inorganic salts. As known,

30   these cultures may be supplemented with corticosteroids,

-18-

such as hydrocortisone at a concentration of 10^{-4} to 10^{-7} M, or other corticosteroids at equal potent dose, such as cortisone, dexamethasome or solumedrol. These cultures are typically carried out at a pH which is roughly physiologic,

5   i.e. 6.9 to 7.4. The medium is typically exposed to an oxygen-containing atmosphere which contains from 4 to 20 vol. percent oxygen, preferably 6 to 8 vol. percent oxygen.

Using these standard culture techniques, the cell mass used may be enriched, by any desired amount, such as by up

10   to 10^3 fold or more, either for stem cell content or for hematopoietic progenitor cell content. Different known methods may be used to achieve this enrichment, corresponding either to a negative selection method or a positive selection method. For example, in accordance with

15   the negative selection method, mature cells are removed using immunological techniques, e.g., labelling non-progenitor, non-stem cells with a panel of mouse anti-human monoclonal antibodies, then removing the mouse antibody-coated cells by adherence to rabbit-anti-mouse Ig-coated

20   plastic dishes. See e.g., Emerson et al, J. Clin. Invest. (1985) 76:1286-1290.

The present invention relies on a fundamental alteration of the conditions of liquid human bone marrow cultures under any of the above conditions; rapid

25   replacement of the nutrient medium. Standard culture schedules call for medium and serum to be exchanged weekly, either as a single exchange performed weekly or a one-half medium and serum exchange performed twice weekly. In accordance with the present invention, the nutrient medium

30   of the culture is replaced, preferably perfused, either continuously or periodically, at a rate of about 1 ml per ml of culture per about 24 to about 48 hour period, for cells cultured at a density of from 2 x 10^6 to 1 x 10^7 cells

-19-

per ml. For cell densities of from 1 x 10^4 to 2 x 10^6 cells
per ml the same medium exchange rate may be used. For cell
densities higher than 10^7 cells per ml, the medium exchange
rate may be increased proportionally to achieve a constant
5   medium and serum flux per cell per unit time.

        Replacement of the nutrient medium in accordance with
    the invention may be carried out in any manner which will
    achieve the result of replacing the medium, e.g., by
    removing an aliquot of spent culture medium and replacing
10   it with a fresh aliquot. The flow of the aliquot being
    added may be by gravity, by pump, or by any other suitable
    means. The flow may be in any direction or multiplicity of
    directions, depending upon the configuration and packing of
    the culture. Preferably, the new medium is added to the
15   culture in a manner such that it contacts the cell mass.
    Most preferably, it is added the culture in a manner
    mimicking *in vivo* perfusion, i.e., it is perfused through
    at least part of the cell mass and up to the whole cell
    mass.

20   Another, optional but important, embodiment of the
    present invention, resides in the addition of hematopoietic
    growth factors, including synthetic hematopoietic growth
    factors, to the rapidly exchanged cultures. In a
    particularly preferred aspect of this embodiment, the
25   cytokines IL-3 and GM-CSF are both added, together, to the
    medium at a rate of from 0.1 to 100 ng/ml/day, preferably
    about 0.5 to 10 ng/ml/day, most preferably 1 to 2
    ng/ml/day. Epo may be added to the nutrient medium in an
    amount of from 0.001 to 10 U/ml/day, preferably 0.05 to
30   0.15 U/ml/day. Mast cell growth factor (MCF, c-kit ligand,
    Steel factor), may be added to the medium in an amount of
    from 1 to 100 ng/ml/day, preferably 10 to 50 ng/ml/day.
    IL-1 (α or β) may also be added in an amount of from 10 to

-20-

100 units/ml per 3 to 5 day period. Additionally, IL-6, G-
CSF, basic fibroblast growth factor, IL-7, IL-8, IL-9, IL-
10, IL-11, PDGF, or EGF to be added, at a rate of from 1 to
100 ng/ml/day.

5   The inventors have discovered that when IL-3, GM-CSF
and Epo are used as described above one obtains lineage
specific development of red blood cells. Alternatively,
when IL-3 and GM-CSF, with or without IL-6 or G-CSF, are
used, the culture preferentially produce granulocytes. The
10   inventors also observed that with the cultures of the
invention T and B lymphocytes are lost over time.

           The metabolic product level in the medium is normally
maintained within a particular range. Glucose
concentration is usually maintained in the range of about 5
15   to 20 mM. Lactate concentration is usually maintained
below 35 mM. Glutamine concentration is generally
maintained in the range of from about 1 to 3 mM. Ammonium
concentration is usually maintained below about 2.4 mM.
These concentrations can be monitored by either periodic or
20   on-line continuous measurements using known methods. See,
e.g., Caldwell et al, J. Cell. Physiol. (1991) 147:344-353.

           The cells which may be cultured in accordance with the
present invention may be any human stem cells or human stem
cell-containing cellular mass, including human peripheral
25   blood mononuclear cells, human bone marrow cells, human
fetal liver cells, embryonic stem cells and/or human cord
blood cells. Each of these cell masses contains human stem
cells and/or human hematopoietic progenitor cells. Other
cellular masses containing human stem cells may also be
30   used in accordance with the invention, including any human
stem cell found in human bone marrow.

-21-

In a preferred embodiment of the invention, the cell
culture may be enriched to augment the human stem cell
content of the cell mass. Such enrichment may achieved as
described above, and, when used in accordance with the
5   invention, provides the first useful means for genetic
therapy via gene transfer into human stem cells, including
human stem cells present in human bone marrow and human
bone marrow stem cells. Stem cells present in human bone
marrow are cells obtainable from human bone marrow,
10   peripheral blood, fetal liver, or human cord blood.

Generally, in this embodiment, a packaging cell line
infected with a retrovirus, or a supernatant obtained from
such a packaging cell line culture, or an other gene
transfer vector, is added to human stem cells cultured in
15   accordance with of the invention to obtain stably
genetically transformed human stem cells. The present
invention provides increased levels of stem cell and human
hematopoietic progenitor cell replication, whereas, by
contrast, prior cultures provided only for human
20   hematopoietic progenitor cell replication at a decreasing
rate (i.e., decaying cultures). The present culture system
provides, for the first time, expansion of cells in
culture, which is required for retroviral infection of
cells. Earlier systems in which retroviral infection was
25   carried out on decaying cultures provided no infection of
earlier cells. The present invention, particularly when it
is practiced together with an enriched stem cell pool, and
even more particularly when it is practiced still further
with the use of hematopoietic growth factors, including
30   synthetic growth factors, provides a very effective means
for obtaining stem cell infection in vitro.

The inventors have discovered that addition of
supernatants containing recombinant retroviruses to the

-22-

cultures results in the introduction of the viruses and the
genes they carry into human (hematopoietic) stem cells.
The progenitor cells which arise by division and
differentiation from these stem cells, and the mature blood
5   cells which arise from further division and differentiation
of these progenitor cells, contain the transfected DNA
throughout the period of the hematopoietic culture *in
vitro*. The inventors have observed that when the
retrovirus is only added at the beginning period of the
10   culture, they obtain transfected progenitors and mature
blood cells which can arise only from stem cells present,
proliferating and stably, genetically transformed in the
beginning of the culture, because no retrovirally infected
cell can infect an adjacent cell. Progenitor cells and
15   more mature cells containing the desired genetic material
have accordingly received the gene from the more primitive
stem cells genetically transformed during the initial
retroviral infection period.

In a preferred embodiment of this aspect of the
20   invention, human hematopoietic cells, either isolated from
bone marrow, peripheral blood, fetal liver, or umbilical
cord blood, are first enriched for the presence of stem
cells by removing more mature blood cells. This is
accomplished by incubating the hematopoietic cells with
25   muring monoclonal antibodies recognizing epitopes on mature
blood cells and bone marrow precursor cells but not stem
cells, and then removing the labelled cells by
immunoadherence to a rabbit-anti-mouse-Ig immunoadsorbent
surface. The resultant lineage negative (Lin⁻) cells are
30   then cultured in the presence of a retrovirus or other gene
transfer vector in accordance with the invention.
Preferably the culture is carried out in the presence of
GM-CSF (preferably 1 mg/ml/day) and IL-3 (preferably 1
mg/ml/day) with or without IL-1 (preferably 50 U/ml/4 day

-23-

period), with or without c-kit ligand (Mast cell growth
factor) (preferably 10 ug/ml/day).

The retroviral infection may be performed by either
including into the culture medium, supernatants (e.g., 5 to
5   20% vol/vol) produced by retroviral packaging cell lines
infected with recombinant retrovirus, during the first 2 to
21, preferably 10 to 14 days of the culture, or by
culturing the Lin⁻ cells directly over the infected
retroviral packaging lines themselves, or by both.

10   Preferably, retroviral supernatants are used, and the
period of incubation in the presence of virus is 12 to 16
days. Also preferably, the packaging cell lines are grown
to near confluency, the medium exchanged, and the cell
lines further incubated for 12 to 15 hours. The medium is
15   then collected and used in the transfection of the human
stem cells. However, this protocol is not strictly
required and any supernatant produced by a retroviral
packaging cell line may be used. Any (known) retroviral
packaging cell lines may be used in accordance with the
20   invention and cultured in accordance with any known
protocol (see, e.g., Wilson et al, Science (1990) 248:1413-
1416 and/or Sullenger et al, Cell (1990) 63:601-608).
Illustrative packaging cell lines include NIH 3T3 cells and
renal carcinoma cell line 5637.

25   Any gene which is inserted into a recombinant
retrovirus together with suitable promoter and enhancer
elements that permit its expression can be incorporated
into human stem and hematopoietic progenitor cells. The
invention provides for the first time conditions that
30   permit stem cell survival and proliferation in these
cultures, permitting the creation of stably transfected,
genetically modified human hematopoietic stem cells in

-24-

these cultures. The terms "stably transformed" and "stably transfected" are used in this text to designate incorporation of exogenous DNA into the human stem cell chromosome(s), made possible by the present invention
5   because it permits exposing dividing human stem cells *ex vivo* to such exogenous DNA.

In accordance with the present invention one obtains cultures in which human hematopoietic progenitor cells are produced by division and differentiation from human stem
10   cells throughout a culture period of at least five months. That is, one obtains a culture which supports stem cell survival and proliferation in culture.

Data obtained by the inventors indicates that medium perfusion rate is a very significant variable in
15   determining the behavior of *ex vivo* human bone marrow cultures. This data showed that when the medium exchange rate was increased from the traditional once per week Dexter rate to a daily medium exchange rate of 7 volumes per week, a significant effect on *ex vivo* hematopoiesis is
20   obtained. In experiments carried out by the inventors, all cultures displayed a significant loss of cells during the first 3 to 4 weeks. Following this decay, the cultures stabilized and the effect of a medium perfusion rate became more pronounced.

25   A 3.5 per week medium exchange rate led to the most prolific cultures and also to cultures of greatest longevity in terms of progenitor cell production. Of particular note, during weeks 4 to 10, the biweekly number of nonadherent cells produced was actually stable or
30   increasing.

Over the entire course of the cultures, the cumulative

-25-

number of cells produced after week 3.5 was almost three-
fold greater than that which is produced under the
traditional Dexter culture protocol. Further, stable
production of progenitor cells is maintained until week 18.

5   Human stromal cells, such as stromal cells found in
human bone marrow, may or may not be present in the
cultures of the invention. In typical cultures, stromal
cells are present in the cell culture in an amount of
approximately 10^{-3} to 10^{-1} (stromal cells/total cells).

10   In another aspect of the invention, the inventors
discovered that the cultures of the invention surprisingly
provide increased metabolism and GM-CSF and IL-6 secretion
from human bone marrow stromal cells. Whereas no GM-CSF is
detected in human bone marrow stromal cells supernatant,
15   rapid medium exchange in accordance with the invention
stimulates human bone marrow stromal cells to secrete 300
centograms/ml/day to 200 picograms/ml/day of GM-CSF.
Secretion of IL-6 by human bone marrow stromal cells is
also increased by rapid medium exchange in accordance with
20   the invention from 1 to 2 ng/ml/day to 2 to 4 ng/ml/day.
This increase is observed both when only the rapid medium
exchange rate of the invention is used, and when the rapid
exchange rate together with the addition of hematopoietic
growth factors is used. On the basis of data obtained by
25   the inventors, the effect of the rapid medium exchange
rates of the invention on human stromal cell production of
cytokines should be observed with human stromal cells in
any complex tissue culture system.

           Illustratively, the medium used in accordance with the
30   invention may comprise three basic components. The first
component is a media component comprised of IMDM, MEM,
DMEM, RPMI 1640, Alpha Medium or McCoy's Medium, or an

equivalent known culture medium component. The second is a serum component which comprises at least horse serum or human serum and may optionally further comprise fetal calf serum, newborn calf serum, and/or calf serum. The third

5   component is a corticosteroid, such as hydrocortisone, cortisone, dexamethasome, solumedrol, or a combination of these, preferably hydrocortisone.

The compositional make up of various media which can be used are set forth below.

-27-

Iscove's Modified Dulbecco's Media (IMDM)[1,2,3]

COMPONENT	380-2440 1X Liquid mg/L	430-2200 Powder mg/L
INORGANIC SALTS:		
CaCl₂ (anhyd.)	165.00	165.00
KCl	330.00	330.00
KNO₃	0.076	0.076
MgSO₄ (anhyd.)	97.67	97.67
NaCl	4505.00	4505.00
NaHCO₃	3024.00	—
NaH₂PO₄·H₂O	125.00	125.00
Na₂SeO₃5H₂O	0.0173	0.0173
OTHER COMPONENTS:		
D-Glucose	4500.00	4500.00
Phenol red	15.00	15.00
HEPES	5958.00	5958.00
Sodium pyruvate	110.00	110.00
AMINO ACIDS:		
L-Alanine	25.00	25.00
L-Asparagine·H₂O	28.40	28.40
L-Arginine·HCl	84.00	84.00
L-Aspartic acid	30.00	30.00
L-Cystine·2HCl	91.24	91.24
L-Glutamic acid	75.00	75.00
L-Glutamine	584.00	584.00
Glycine	30.00	30.00
L-Histidine·HCl·H₂O	42.00	42.00
L-Isoleucine	105.00	105.00
L-Leucine	105.00	105.00
L-Lysine·HCl	146.00	146.00
L-Methionine	30.00	30.00
L-Phenylalanine	66.00	66.00
L-Proline	40.00	40.00
L-Serine	42.00	42.00
L-Threonine	95.00	95.00
L-Tryptophan	16.00	16.00
L-Tyrosine·2Na·2H₂O	103.79	103.79
L-Valine	94.00	94.00

COMPONENT	380-2440 1X Liquid mg/L	430-2200 Powder mg/L
VITAMINS:		
Biotin	0.013	0.013
D-Ca pantothenate	4.00	4.00
Choline chloride	4.00	4.00
Folic acid	4.00	4.00
i-Inositol	7.20	7.20
Niacinamide	4.00	4.00
Pyridoxal·HCl	4.00	4.00
Riboflavin	0.40	0.40
Thiamine·HCl	4.00	4.00
Vitamin B₁₂	0.013	0.013

1. Dulbecco, R. and Freeman, G. (1959) Virology 8, 396. Smith, J. D., Freeman, G., Vogt, M., and Dulbecco, R. (1960) Virology 12, 185. Tissue Culture Standards Committee, In Vitro 6:2, 93.
2. Iscove, N. N. and Melchers, F., J. Experimental Medicine 147, 923.
a. Values shown are in conformance with Tissue Culture Standards Committee, In Vitro (1970) 9:6.
3. Iscove, N. N., personal communication.

Dulbecco's[1] Modified Eagle Media (D-MEM)

COMPONENT	320-1825 1X Liquid mg/L	300-2320 1X Liquid mg/L	430-1600 Powder mg/L	320-1965 1X Liquid mg/L	360-3430 1X Liquid mg/L	430-2100/430-2900 Powder mg/L	430-3000 Powder mg/L	Powder mg/L	320-1940 1X Liquid mg/L	320-1970 1X Liquid mg/L	320-1995 1X Liquid mg/L	430-3700 Powder mg/L	320-1945 1X Liquid mg/L	430-3000 Powder mg/L
INORGANIC SALTS:														
CaCl₂ (anhyd.)	200.00	200.00	200.00	200.00	200.00	200.00	200.00	200.00	200.00	200.00	200.00	200.00	200.00	200.00
Fe(NO₃)₃·9H₂O	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
KCl	400.00	400.00	400.00	400.00	400.00	400.00	400.00	400.00	400.00	400.00	400.00	400.00	400.00	400.00
MgSO₄ (anhyd.)	—	—	97.67	—	—	97.67	97.67	97.67	—	—	—	97.67	—	97.67
MgSO₄·7H₂O	200.00	200.00	—	200.00	200.00	—	—	—	200.00	200.00	200.00	—	200.00	—
NaCl	6400.00	4750.00	6400.00	6400.00	4750.00	6400.00	6400.00	6400.00	6400.00	6400.00	6400.00	4750.00	6400.00	6400.00
NaHCO₃	3700.00	3700.00	—	3700.00	3700.00	—	—	—	3700.00	3700.00	3700.00	—	3700.00	—
NaH₂PO₄·H₂O*	125.00	125.00	125.00	125.00	125.00	125.00	125.00	125.00	125.00	125.00	125.00	125.00	125.00	125.00
OTHER COMPONENTS:														
D-Glucose	1000.00	1000.00	1000.00	4500.00	4500.00	4500.00	4500.00	4500.00	4500.00	4500.00	4500.00	4500.00	4500.00	—
Phenol red	15.00	15.00	15.00	15.00	15.00	15.00	15.00	—	15.00		15.00	15.00	15.00	—
HEPES	—	5958.00	—	—	5958.00	—	—	—	—	—	—	5958.00	—	—
Sodium pyruvate	110.00	110.00	110.00	—	—	—	110.00	—	—	—	110.00	—	—	—
AMINO ACIDS:														
L-Arginine·HCl	84.00	84.00	84.00	84.00	84.00	84.00	84.00	84.00	84.00	84.00	84.00	84.00	84.00	84.00
L-Cystine	48.00	48.00	—	48.00	48.00	—	—	—	48.00	48.00	48.00	—	48.00	—
L-Cystine·2HCl	—	—	62.57	—	—	62.57	62.57	62.57	—	—	—	62.57	—	62.57
L-Glutamine	584.00	584.00	584.00	584.00	584.00	584.00	584.00	584.00	—	—	584.00	584.00	584.00	—
Glycine	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00
L-Histidine·HCl·H₂O	42.00	42.00	42.00	42.00	42.00	42.00	42.00	42.00	42.00	42.00	42.00	42.00	42.00	42.00
L-Isoleucine	105.00	105.00	105.00	105.00	105.00	105.00	105.00	105.00	105.00	105.00	105.00	105.00	105.00	105.00
L-Leucine	105.00	105.00	105.00	105.00	105.00	105.00	105.00	105.00	105.00	105.00	105.00	105.00	105.00	105.00
L-Lysine·HCl	146.00	146.00	146.00	146.00	146.00	146.00	146.00	146.00	146.00	146.00	146.00	146.00	146.00	146.00
L-Methionine	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00	—	30.00	30.00	30.00	30.00
L-Phenylalanine	66.00	66.00	66.00	66.00	66.00	66.00	66.00	66.00	66.00	66.00	66.00	66.00	66.00	66.00
L-Serine	42.00	42.00	42.00	42.00	42.00	42.00	42.00	42.00	42.00	42.00	42.00	42.00	42.00	42.00
L-Threonine	95.00	95.00	95.00	95.00	95.00	95.00	95.00	95.00	95.00	95.00	95.00	95.00	95.00	95.00
L-Tryptophan	16.00	16.00	16.00	16.00	16.00	16.00	16.00	16.00	16.00	16.00	16.00	16.00	16.00	16.00
L-Tyrosine	72.00	72.00	—	72.00	72.00	—	—	—	72.00	72.00	72.00	—	72.00	—
L-Tyrosine·2Na·2H₂O	—	—	103.79	—	—	103.79	103.79	103.79	—	—	—	103.79	—	103.79
L-Valine	94.00	94.00	94.00	94.00	94.00	94.00	94.00	94.00	94.00	94.00	94.00	94.00	94.00	94.00
VITAMINS:														
D-Ca pantothenate	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Choline chloride	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Folic acid	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
i-Inositol	7.20	7.20	7.20	7.20	7.20	7.20	7.20	7.20	7.20	7.20	7.20	7.20	—	7.20
Niacinamide	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Pyridoxal·HCl	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Riboflavin	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
Thiamine·HCl	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00

.. Dulbecco, R. and Freeman, G. (1959) Virology 8, 396. Smith, J.D., Freeman, G., Vogt, M., and Dulbecco, R. (1960) Virology 12, 185. Tissue Culture Standards Committee, In Vitro 6, 2, 93.

[1] Values shown are in conformance with the Tissue Culture Standards Committee, In Vitro (1970) 9:6.

Minimum Essential Media (MEM)[1]

COMPONENT	320-2561[2] 1X Liquid	410-3000 Powder	320-2571[3] 1X Liquid	410-1900 Powder	320-2570 1X Liquid	330-1090 1X Liquid	380-2360 1X Liquid	330-1430 1X Liquid	410-1700 Powder	320-1890 1X Liquid	330-1096 1X Liquid	410-2400 Powder	320-1097 1X Liquid	410-2500 Powder
	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
INORGANIC SALTS:														
CaCl₂ (anhyd.)	200.00	200.00	200.00	200.00	200.00	200.00	200.00	2000.00	200.00	200.00	200.00	200.00	200.00	200.00
KCl	400.00	400.00	400.00	400.00	400.00	400.00	400.00	4000.00	400.00	400.00	400.00	400.00	400.00	400.00
MgSO₄ (anhyd.)	—	97.67	—	97.67	—	—	—	—	97.67	—	—	97.67	—	97.67
MgSO₄·7H₂O	200.00	—	200.00	—	200.00	200.00	200.00	2000.00	—	200.00	200.00	—	200.00	—
NaCl	6800.00	6800.00	6800.00	6800.00	6800.00	6800.00	6350.00	68000.00	6800.00	6800.00	6800.00	6800.00	6800.00	6800.00
NaHCO₃	2200.00	—	2200.00	—	2200.00	2200.00	2200.00	—	—	2200.00	1500.00	—	2200.00	—
NaH₂PO₄·H₂O	140.00	140.00	140.00	140.00	140.00	140.00	140.00	1400.00	140.00	140.00	140.00	140.00	—	—
OTHER COMPONENTS:														
D-Glucose	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	10000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00
HEPES	—	—	—	—	—	—	5958.00	—	—	—	—	—	—	—
Lipoic acid	0.20	0.20	0.20	0.20	—	—	—	—	—	—	—	—	—	—
Phenol red	10.00	10.00	10.00	10.00	10.00	10.00	10.00	100.00	6.00	10.00	10.00	10.00	10.00	10.00
Sodium pyruvate	110.00	110.00	110.00	110.00	—	—	—	—	—	—	—	—	—	—
Sodium succinate	—	—	—	—	—	—	—	—	100.00	—	—	—	—	—
Succinic acid	—	—	—	—	—	—	—	—	75.00	—	—	—	—	—
AMINO ACIDS:														
L-Alanine	25.00	25.00	25.00	25.00	—	—	—	—	—	—	—	—	—	—
L-Arginine	105.00	—	105.00	—	—	—	—	—	—	—	—	—	—	—
L-Arginine·HCl	—	126.64	—	126.64	126.00	126.00	126.00	1260.00	126.00	126.00	126.00	126.00	126.00	126.00
L-Asparagine·H₂O	50.00	50.00	50.00	50.00	—	—	—	—	—	—	—	—	—	—
L-Aspartic acid	30.00	30.00	30.00	30.00	—	—	—	—	—	—	—	—	—	—
L-Cystine	24.00	—	24.00	—	24.00	24.00	24.00	240.00	—	24.00	—	—	24.00	—
L-Cystine·2HCl	—	31.28	—	31.28	—	—	—	—	31.00	—	31.00	31.29	—	31.29
L-Cysteine·HCl·H₂O	100.00	100.00	100.00	100.00	—	—	—	—	—	—	—	—	—	—
L-Glutamic acid	75.00	75.00	75.00	75.00	—	—	—	—	—	—	—	—	—	—
L-Glutamine	292.00	292.00	292.00	292.00	292.00	—	—	—	—	—	—	292.00	292.00	292.00
Glycine	50.00	50.00	50.00	50.00	—	—	—	—	—	—	—	—	—	—
L-Histidine	31.00	—	31.00	—	—	—	—	—	—	—	—	—	—	—
L-Histidine·HCl·H₂O	—	42.00	—	42.00	42.00	42.00	42.00	430.00	42.00	42.00	42.00	42.00	42.00	42.00
L-Isoleucine	52.40	52.40	52.40	52.40	52.40	52.00	52.00	520.00	52.00	52.00	52.00	52.00	52.00	52.00
L-Leucine	52.40	52.40	52.40	52.40	52.40	52.00	52.00	520.00	52.00	—	52.00	—	52.00	52.00
L-Lysine	58.00	—	58.00	—	—	—	—	—	—	—	—	—	—	—
L-Lysine·HCl	—	72.50	—	72.50	72.50	72.50	72.50	725.00	72.50	72.50	72.50	—	72.50	72.50
L-Methionine	15.00	15.00	15.00	15.00	15.00	15.00	15.00	150.00	15.00	15.00	15.00	—	15.00	15.00
L-Phenylalanine	32.00	32.00	32.00	32.00	32.00	32.00	32.00	320.00	32.00	32.00	32.00	32.00	32.00	32.00
L-Proline	40.00	40.00	40.00	40.00	—	—	—	—	—	—	—	—	—	—
L-Serine	25.00	25.00	25.00	25.00	—	—	—	—	—	—	—	—	—	—
L-Threonine	48.00	48.00	48.00	48.00	48.00	48.00	48.00	480.00	48.00	48.00	48.00	48.00	48.00	48.00
L-Tryptophan	10.00	10.00	10.00	10.00	10.00	10.00	10.00	100.00	10.00	10.00	10.00	10.00	10.00	10.00
L-Tyrosine	36.00	—	36.00	—	36.00	36.00	36.00	360.00	36.00	36.00	—	—	36.00	—
L-Tyrosine·2Na·2H₂O	—	51.90	—	51.90	—	—	—	—	—	—	51.90	51.90	—	51.90
D-Valine	—	—	—	—	92.00	—	—	—	—	—	—	—	—	—
L-Valine	46.00	46.00	46.00	46.00	—	46.00	46.00	460.00	46.00	46.00	46.00	46.00	46.00	46.00

Minimum Essential Media (MEM)[1]—Continued

COMPONENT	320-2561[a] 1X Liquid mg/L	410-2080[a] Powder mg/L	320-2571[a] 1X Liquid mg/L	410-1900[a] Powder mg/L	320-2570 1X Liquid mg/L	320-1090 1X Liquid mg/L	380-2560 1X Liquid mg/L	330-1430 10X Liquid mg/L	410-1700 Powder mg/L	320-1890 1X Liquid mg/L	320-1890 Powder mg/L	320-4060 410-3600 1X Liquid mg/L	Powder mg/L	320-1007 1X Liquid mg/L	410-2560 Powder mg/L
VITAMINS:															
L-Ascorbic acid	50.00	50.00	50.00	50.00	—	—	—	—	—	—	—	—	—	—	—
Biotin	0.10	0.10	0.10	0.10	—	—	—	—	—	—	—	—	—	—	—
D-Ca pantothenate	1.00	1.00	1.00	1.00	1.00	1.00	1.00	10.00	1.00	1.00	1.00	1.00	1.00	1.00	
Choline bitartrate	—	—	—	—	—	—	—	—	1.80	—	—	—	—	—	
Choline chloride	1.00	1.00	1.00	1.00	1.00	1.00	1.00	10.00	1.00	1.00	1.00	1.00	1.00	1.00	
Folic acid	1.00	1.00	1.00	1.00	1.00	1.00	1.00	10.00	1.00	1.00	1.00	1.00	1.00	1.00	
i-Inositol	2.00	2.00	2.00	2.00	2.00	2.00	2.00	20.00	2.00	2.00	2.00	2.00	2.00	2.00	
Niacinamide	1.00	1.00	1.00	1.00	1.00	1.00	1.00	10.00	1.00	1.00	1.00	1.00	1.00	1.00	
Pyridoxal-HCl	1.00	1.00	1.00	1.00	1.00	1.00	1.00	10.00	1.00	1.00	1.00	1.00	1.00	1.00	
Riboflavin	0.10	0.10	0.10	0.10	0.10	0.10	0.10	1.00	0.10	0.10	0.10	0.10	0.10	0.10	
Thiamine-HCl	1.00	1.00	1.00	1.00	1.00	1.00	1.00	10.00	1.00	1.00	1.00	1.00	1.00	1.00	
Vitamin B12	1.36	1.36	1.36	1.36	—	—	—	—	—	—	—	—	—	—	
RIBONUCLEOSIDES:															
Adenosine	—	—	10.00	10.00	—	—	—	—	—	—	—	—	—	—	
Cytidine	—	—	10.00	10.00	—	—	—	—	—	—	—	—	—	—	
Guanosine	—	—	10.00	10.00	—	—	—	—	—	—	—	—	—	—	
Uridine	—	—	10.00	10.00	—	—	—	—	—	—	—	—	—	—	
DEOXYRIBONUCLEOSIDES:															
2' Deoxyadenosine	—	—	10.00	10.00	—	—	—	—	—	—	—	—	—	—	
2' Deoxycytidine-HCl	—	—	11.00	11.00	—	—	—	—	—	—	—	—	—	—	
2' Deoxyguanosine	—	—	10.00	10.00	—	—	—	—	—	—	—	—	—	—	
Thymidine	—	—	10.00	10.00	—	—	—	—	—	—	—	—	—	—	

1. Eagle, H. (1959) *Science*. *130*, 432.
2. *Nature*. *New Biology* (1971) *230*, 310.
a. Original formula lists this component as $NaH_2PO_4 \cdot 2H_2O$.

RPMI Media 1640[1]

COMPONENT	320-1870 1X Liquid	330-1875 1X Liquid	330-2511 10X Liquid	380-2400 1X Liquid	430-1800 Powder	430-3200 Powder	430-3400 Powder	320-1835 1X Liquid	320-1877 1X Liquid
	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
INORGANIC SALTS:									
Ca(NO$_3$)$_2$·4H$_2$O	100.00	100.00	1000.00	100.00	100.00	100.00	100.00	100.00	100.00
KCl	400.00	400.00	4000.00	400.00	400.00	400.00	400.00	400.00	400.00
MgSO$_4$ (anhyd.)	—	—	—	—	48.84	48.84	48.84	—	—
MgSO$_4$·7H$_2$O	100.00	100.00	1000.00	100.00	—	—	—	100.00	100.00
NaCl	6000.00	6000.00	60000.00	5300.00	6000.00	6000.00	5850.00	6000.00	6000.00
NaHCO$_3$	2000.00	2000.00	—	2000.00	—	—	—	2000.00	2000.00
Na$_2$HPO$_4$ (anhyd.)	—	—	—	—	800.00	800.00	800.00	—	—
Na$_2$HPO$_4$·7H$_2$O	1512.00	1512.00	15120.00	1512.00	—	—	—	1512.00	—
OTHER COMPONENTS:									
D-Glucose	2000.00	2000.00	20000.00	2000.00	2000.00	2000.00	2000.00	2000.00	2000.00
Glutathione (reduced)	1.00	1.00	10.00	1.00	1.00	1.00	1.00	1.00	1.00
HEPES	—	—	—	5958.00	—	—	5957.50	—	—
Phenol red	5.00	5.00	50.00	5.00	5.00	—	5.00	—	5.00
AMINO ACIDS:									
L-Arginine	200.00	200.00	2000.00	200.00	200.00	200.00	200.00	200.00	200.00
L-Asparagine	50.00	50.00	500.00	50.00	50.00	50.00	50.00	50.00	50.00
L-Aspartic acid	20.00	20.00	200.00	20.00	20.00	20.00	20.00	20.00	20.00
L-Cystine	50.00	50.00	500.00	50.00	—	—	—	50.00	50.00
L-Cystine·2HCl	—	—	—	—	65.15	65.15	65.15	—	—
L-Glutamic acid	20.00	20.00	200.00	20.00	20.00	20.00	20.00	20.00	20.00
L-Glutamine	—	300.00	3000.00	300.00	300.00	300.00	300.00	300.00	300.00
Glycine	10.00	10.00	100.00	10.00	10.00	10.00	10.00	10.00	10.00
L-Histidine	15.00	15.00	150.00	15.00	15.00	15.00	15.00	15.00	15.00
L-Hydroxyproline	20.00	20.00	200.00	20.00	20.00	20.00	20.00	20.00	20.00
L-Isoleucine	50.00	50.00	500.00	50.00	50.00	50.00	50.00	50.00	50.00
L-Leucine	50.00	50.00	500.00	50.00	50.00	50.00	50.00	50.00	50.00
L-Lysine·HCl	40.00	40.00	400.00	40.00	40.00	40.00	40.00	40.00	40.00
L-Methionine	15.00	15.00	150.00	15.00	15.00	15.00	15.00	15.00	15.00
L-Phenylalanine	15.00	15.00	150.00	15.00	15.00	15.00	15.00	15.00	15.00
L-Proline	20.00	20.00	200.00	20.00	20.00	20.00	20.00	20.00	20.00
L-Serine	30.00	30.00	300.00	30.00	30.00	30.00	30.00	30.00	30.00
L-Threonine	20.00	20.00	200.00	20.00	20.00	20.00	20.00	20.00	20.00
L-Tryptophan	5.00	5.00	50.00	5.00	5.00	5.00	5.00	5.00	5.00
L-Tyrosine	20.00	20.00	200.00	20.00	—	—	—	20.00	20.00
L-Tyrosine·2Na·2H$_2$O	—	—	—	—	28.83	28.83	28.83	—	—
L-Valine	20.00	20.00	200.00	20.00	20.00	20.00	20.00	20.00	20.00
VITAMINS:									
Biotin	0.20	0.20	2.00	0.20	0.20	0.20	0.20	0.20	0.20
D-Ca pantothenate	0.25	0.25	2.50	0.25	0.25	0.25	0.25	0.25	0.25
Choline chloride	3.00	3.00	30.00	3.00	3.00	3.00	3.00	3.00	3.00
Folic acid	1.00	1.00	10.00	1.00	1.00	1.00	1.00	1.00	1.00
i-Inositol	35.00	35.00	350.00	35.00	35.00	35.00	35.00	35.00	35.00
Niacinamide	1.00	1.00	10.00	1.00	1.00	1.00	1.00	1.00	1.00
Para-aminobenzoic acid	1.00	1.00	10.00	1.00	1.00	1.00	1.00	1.00	1.00
Pyridoxine·HCl	1.00	1.00	10.00	1.00	1.00	1.00	1.00	1.00	1.00
Riboflavin	0.20	0.20	2.00	0.20	0.20	0.20	0.20	0.20	0.20
Thiamine·HCl	1.00	1.00	10.00	1.00	1.00	1.00	1.00	1.00	1.00
Vitamin B$_{12}$	0.005	0.005	0.05	0.005	0.005	0.005	0.005	0.005	0.005

[1] Moore, G.E., Gerner, R.E. and Franklin, H.A. (1967) J.A.M.A. 199, 519.

McCoy's 5A Media (modified)[1,2,3]

COMPONENT	320-6600 1X Liquid	390-2330 1X Liquid	430-1500 Powder	320-6600 1X Liquid	320-6601* 1X Liquid	320-6610 1X Liquid	320-6620 1X Liquid	320-6630 1X Liquid
	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
INORGANIC SALTS:								
$CaCl_2$ (anhyd.)	100.00	100.00	100.00	—	140.00	100.00	100.00	100.00
KCl	400.00	400.00	400.00	400.00	400.00	400.00	400.00	400.00
KH_2PO_4	—	—	—	—	60.00	—	—	—
$MgCl_2 \cdot 6H_2O$	—	—	—	—	100.00	—	—	—
$MgSO_4$ (anhyd.)	—	—	97.67	—	—	—	—	—
$MgSO_4 \cdot 7H_2O$	200.00	200.00	—	200.00	100.00	200.00	200.00	200.00
NaCl	6460.00	5100.00	6460.00	6460.00	8000.00	6460.00	6460.00	6460.00
$NaHCO_3$	2200.00	2200.00	—	2200.00	350.00	2200.00	2200.00	2200.00
$NaH_2PO_4 \cdot H_2O$	580.00	580.00	580.00	1400.00	—	580.00	580.00	580.00
$Na_2HPO_4 \cdot 7H_2O$	—	—	—	—	90.00	—	—	—
OTHER COMPONENTS:								
Bacto-peptone	600.00	600.00	600.00	600.00	600.00	600.00	600.00	600.00
Fetal Bovine Serum	—	—	—	—	—	c	c	c
D-Glucose	3000.00	3000.00	3000.00	3000.00	1000.00	3000.00	3000.00	3000.00
Glutathione (reduced)	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
HEPES	—	5958.00	—	—	—	—	—	—
Phenol red	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
AMINO ACIDS:								
L-Alanine	13.90	13.90	13.90	13.90	13.90	13.90	13.90	13.90
L-Arginine·HCl	42.10	42.10	42.10	42.10	42.10	42.10	42.10	42.10
L-Asparagine*	45.00	45.00	45.00	45.00	45.00	45.00	45.00	45.00
L-Aspartic acid	19.97	19.97	19.97	19.97	19.97	19.97	19.97	19.97
L-Cystine*	31.50	31.50	31.50	31.50	31.50	31.50	31.50	31.50
L-Glutamic acid	22.10	22.10	22.10	22.10	22.10	22.10	22.10	22.10
L-Glutamine	219.20	219.20	219.20	219.20	219.20	219.20	219.20	219.20
Glycine	7.50	7.50	7.50	7.50	7.50	7.50	7.50	7.50
L-Histidine·HCl·H₂O	20.96	20.96	20.96	20.96	20.96	20.96	20.96	20.96
L-Hydroxyproline	19.70	19.70	19.70	19.70	19.70	19.70	19.70	19.70
L-Isoleucine	39.36	39.36	39.36	39.36	39.36	39.36	39.36	39.36
L-Leucine	39.36	39.36	39.36	39.36	39.36	39.36	39.36	39.36
L-Lysine·HCl	36.50	36.50	36.50	36.50	36.50	36.50	36.50	36.50
L-Methionine	14.90	14.90	14.90	14.90	14.90	14.90	14.90	14.90
L-Phenylalanine	16.50	16.50	16.50	16.50	16.50	16.50	16.50	16.50
L-Proline	17.30	17.30	17.30	17.30	17.30	17.30	17.30	17.30
L-Serine	26.30	26.30	26.30	26.30	26.30	26.30	26.30	26.30
L-Threonine	17.90	17.90	17.90	17.90	17.90	17.90	17.90	17.90
L-Tryptophan	3.10	3.10	3.10	3.10	3.10	3.10	3.10	3.10
L-Tyrosine	18.10	18.10	—	18.10	18.10	18.10	18.10	18.10
L-Tyrosine·2Na·2H₂O	—	—	26.10	—	—	—	—	—
L-Valine	17.60	17.60	17.60	17.60	17.60	17.60	17.60	17.60

McCoy's 5A Media (modified)[1,2,3]—Continued

COMPONENT	320-6600 1X Liquid	380-2330 1X Liquid	430-1500 Powder	320-6608 1X Liquid	320-6601[4] 1X Liquid	320-6610 1X Liquid	320-6620 1X Liquid	320-6630 1X Liquid
	mg/l	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
VITAMINS:								
Ascorbic acid	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Biotin	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Choline chloride	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
D-Ca pantothenate	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Folic acid	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
i-Inositol	36.00	36.00	36.00	36.00	36.00	36.00	36.00	36.00
Niacinamide	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Nicotinic acid	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Para-aminobenzoic acid	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Pyridoxal-HCl	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Pyridoxine-HCl	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Riboflavin	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Thiamine-HCl	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Vitamin B12	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00

1. McCoy, T.A., Maxwell, M., and Kruse, P.F. (1959) Proc. Soc. Exper. Biol. Med. 100, 115.
2. Hsu, T.C. and Kellogg, D.S., Jr. (1960) J. Nat. Cancer Inst. 25, 221.
3. Iwakata, S. and Grace, J.T., Jr. (1964) N.Y.J. Med. 64:18, 2279.
4. McCoy's 5A Medium formulated with Hanks' and Suspension Salts is a GIBCO modification and is not cited in references 1-3
5. HCl form listed by the Tissue Culture Standards Committee. In Vitro (1974) 9:6.
6. Monohydrate form listed by the Tissue Culture Standards Committee. In Vitro (1974) 9:6.
7. Fetal Bovine Serum Supplementation: Cat. No.   FBS
                               320-6610   10% v/v
                               320-6620   20% v/v
                               320-6630   30% v/v

-34-

The serum component may be present in the culture in an amount of at least 1% (v/v) to 50% (v/v). The serum concentration may be preferably in the neighborhood of 15 to 30% (v/v). For higher serum concentrations, the exchange rate is increased proportionately. The third component may be present in an amount of from 10^{-7} M to 10^{-4} M, and is preferably present in an amount of from 5×10^{-6} to 5×10^{-5} M. The media component represents the balance such that all three components add up to 100%. Alternatively the serum component can be replaced by any of several standard serum replacement mixtures which typically include insulin, albumin, and lecithin or cholesterol. See, Migliaccio et al, Exp. Hematol. (1990) 18:1049-1055, Iscove et al, Exp. Cell Res. (1980) 126:121-126, and Dainiak et al, J. Clin. Invest. (1985) 76:1237-1242.

Illustratively, human hematopoietic stem cell concentration may be increased as follows. Red blood cells are removed from a bone marrow aspirate by ficol-hypaque gradient centriguration. The mononuclear cells are then incubated with a "cocktail" of antibodies which recognize mature blood elements including red blood cells and red blood cell, granulocytes, macrophages, and mature lymphocytes (both B- and T-cells). In addition, antibodies are included which recognize committed progenitor cells (including anti CD 33). The mature cells are then removed by one of various procedures including panning, magnetic beads, or cell sprting on a Fluorescent Activated Cell Sorter (FACS). By removing the mature elements, the chance of recombinant virus infection, and therefore transfer of the desired gene(s), to hematopoietic stem cells is greatly facilitated.

In another embodiment, methods are provided for the growth of hematopoietic cells in culture, employing

-35-

fibroblast cells, normally transformed, for providing
growth factors, with proteinaceous components added to the
mixtures of the fibroblast cells and hematopoietic cells,
and substantially continuous perfusion, optionally with
5   recycling, to maintain an effective growth environment.

The description of the method of this embodiment
therefore may be divided into descriptions of the perfusion
conditions, the reactor and its internal structure, and the
transformed fibroblasts.

10   The reactor comprises a vessel which may be of any
convenient shape which allows for the necessary cell
distribution, introduction of nutrients and oxygen, removal
of waste metabolic products, optional recycling of
hematopoietic cells, substitution of stromal cells, and
15   harvesting of hematopoietic cells. The reactor should
provide for conditions which substantially mimic bone
perfusion. *In vivo*, about 0.08 ml of serum per ml of bone
marrow per minute is perfused. This translates into about
0.3 ml of serum per 10^6 cells per day. The media will
20   therefore be changed on the average at least 50%,
preferably at least 100%, in any 24 hour period, so as to
maintain a level of metabolic products which is not growth
limiting. The rate of change will generally
be from about 0.5 to 1.0 ml of perfusion medium per 10^6
25   cells per day, empirically mimicking *in vivo* perfusion
rates.

The rate of perfusion in the bioreactor will vary
depending on the cell density in the reactor. For cells
cultured at 2-10 x 10^6 cells/ml, this rate is 1 ml/ml
30   reactor volume per 24-48 hours, where the medium used
contains 20% serum, either 10% fetal calf serum and 10%
horse serum, or 20% fetal calf serum. For higher cell

densities, the perfusion rate will be increased
proportionately to achieve a constant serum flux per cell
per time. Thus if the cells are cultured at 5 x 10^8 cell/ml
the perfusion rate will be 0.1 ml/ml reactor volume per
5   minute. These flow rates, matching serum and medium flux
rates to cell density, are essential to stimulating the
endogenous production of hematopoietic growth factors from
the normal human bone marrow stromal cells in the culture.
The hematopoietic growth factors induced by these serum and
10   medium flux rates include GM-CSF, and may also include
S-CSF, IL-6 and G-CSF as well as other hematopoietic growth
factors. These rates will be established in the
bioreactors such that the shear stress from longitudinal
flow experienced by the stem cells and progenitor cells at
15   their stromal cell attachment sites are between 1.0 and 5.0
dynes/square cm.

Various media may be employed for the growth of
hematopoietic and stromal cells. Illustrative media
include MEM, IMDM, and RPMI, which may be supplemented by
20   combinations of 5-20% fetal calf serum, 5-20% calf serum,
and 0-15% horse serum, and/or serum free media supplemented
with PDGF, EGF, FGF, HGF or other growth factors to
stimulate stromal cells or stem cells. To supplement the
growth factors provided by the transformed fibroblasts,
25   additional growth factors may be included in the perfusion
medium, particularly where dedicated cells of a particular
lineage are desired. Among the growth factors which may be
included in the perfusion medium, either by stromal cell
secretion or addition, are GM-CSF, G-CSF, or M-CSF,
30   interleukins 1-7, particularly 1, 3, 6, and 7, TGF-α or β,
erythropoietin, or the like, particularly human factors.
Of particular interest is the presence of about 0.5-2,
preferably 1, ng/ml G-MCSF, and 0.5-2, preferably 1 ng/ml,
as well as a 0.1-2 U/ml/day of final concentration of

erythropoietin, from about 100-300 ng/ml/day of G-CSF and
about 1-10 ng/ml/day of stem cell growth factor (S-CSF,
also referenced as Mast Cell Growth Factor or Kit ligand).
It is understood that one or more, preferably at least two
5   of the growth factors will be provided by secretion from
transformed cells, which will be present in an amount
sufficient to maintain the desired level of the growth
factors in the perfusion medium.

        Conveniently, in the reactor, physiologic temperature
10   will be employed, namely 37°C, although lower temperatures
may also be employed, including 33°, usually not being
below 25°C. Humidity will generally be about 100%, where
the air will contain about 5% carbon dioxide. The
perfusion medium may be oxygenated external to the reactor
15   or internal to the reactor, various means being provided
for internal oxygenation. Internal oxygenation may be
achieved with hollow fibers, porous sintered disks,
silicone tubing or other membranes of suitable porosity and
hydrophobicity. The nutrient level and metabolic product
20   level will normally be maintained in a relatively narrow
range. Glucose level will usually be in the range of about
5 to 20 mM, usually about 10 to 20 mM, lactate
concentration will usually be maintained below about 35 mM
and may be allowed to be over 20 mM. Glutamine
25   concentration will generally be maintained in the range of
about 1 to 3 mM, usually 1.5 to 2.5 mM, while ammonia
concentration will usually be maintained below about 2.5
mM, preferably below about 2.0 mM.

        The flow of fluid may be by gravity, by a pump, or
30   other means, where the flow may be in any direction or a
multiplicity of directions, depending upon the nature of
the packing in the reactor. Desirably, laminar flow may be
employed where the flow may be substantially horizontal

across the reactor or vertical flow may be employed, where the flow is from the bottom to the top of the reactor or vice-versa.

Where the source of human hematopoietic cells is
5   suspected of having neoplastic cells, e.g., leukemic lymphoma or carcinoma, the perfusion flow can be selected so as to segregate the normal progenitor cells from the neoplastic hematopoietic cells. It is found that normal hematopoietic progenitor cells adhere to stroma and matrix
10 proteins with an affinity able to withstand approximately 1.5-2.0 dynes/cm^2 stress from longitudinal fluid flow. By contrast, neoplastic cells and their progenitors have a substantially weaker affinity for stroma, in the range of about 0.05-1.2 dynes/cm^2. By providing for a perfusion
15 flow rate which provides sheer stress rates intermediate between that tolerated by normal and neoplastic progenitor cells, generally greater than 1 dyne/cm^2, one can provide for separation of the neoplastic progenitor cells from the normal progenitor cells, generally maintaining the
20 perfusion for at least about two days, preferably at least about five days, and more preferably seven days or more. In this manner, one can expand normal hematopoietic cells from a human patient, while at the same time using the appropriate flow rates, separate neoplastic cells. In this
25 manner, one can provide for autologous hematopoietic cells from a patient suffering from neoplasia, expand the normal hematopoietic cells during a period of treatment of the patient by chemotherapy or X-irradiation, and then restore normal hematopoietic cells to the patient to restore
30 hematopoiesis and the immune system of the patient.

Illustrative of the use of shear stress to separate hematopoietic tumor cells from normal hematopoietic cells is the situation of chronic myelogenous leukemia (CML).

Shear stress tolerance for CML cells is in the range of
0.05-1.2 dyne/cm^2. This difference permits the efficient
removal of CML cells with an individual bone marrow sample.
By employing a shear rate of about 1.2-1.5, preferably 1.3,

5   dynes/cm^2, the CML cell may be efficiently separated.

The shear stress tolerance within an individual's bone
marrow cells may be determined using a tapered radial flow
chamber. In the radial flow chamber, the shear stress
experienced by the cell decreases with distance "d" from

10   the start of the chamber as a function of 1/d. Bands may
then be analyzed for cell population and the shear stress
set for the desired cell population to be retained.

For the removal of leukemic stem cells, progenitor
cells and stem cells from bone marrow samples from patients

15   with leukemia are first placed into a radial flow chamber.
The radial flow chamber consists of two parallel plates,
made of polycarbonate or glass, which permit the adhesion
of bone marrow stromal cells to the lower plates. The
initial measurements can be performed by either 1)

20   establishing a preformed confluent monolayer of bone marrow
stromal cells prior to hematopoietic cell infusion and then
initiating fluid flow after 12-24 hours, or 2) inoculating
the patient's bone marrow directly into the flow chamber
without using a preformed stromal monolayer, and then

25   waiting 3-4 days before establishing the fluid flow,
usually 0.05-1.0 cc/min. The plates are sealed together at
the edges through a rubber gasket, and held together with
adjustable screws. At the narrow, infusion, end of the
chamber a tube brings fluid into the chamber from a

30   reservoir delivered by a constant pressure syringe-type
pump. At the wide, collection end, the fluid and removed
cells are collected through a separate tube (see Figs. 3
and 3b). After the period of perfusion (usually 3-7 days),

the nonadherent cells are removed, and the plates are
separated, cells from each of 3-5 regions are separately
removed by aspiration and rubber policeman, and each
fraction is analyzed for the presence of leukemic cells by
5   standard techniques (usually karyotypic analysis by
chromosomal banding). Comparison of the leukemic analyses
of each fraction demonstrates in which fraction (i.e. at
which shear stress), the leukemic cells fail to adhere to
the stroma and are removed. In these chambers, the shear
10   stress perceived by the cells declines exponentially as a
function of the distance are from the inlet. (See Fig.
3c). Typically, the nonadherent cells are all or nearly
all leukemic, whereas cells adhering at the in the
narrowest 1/2 of the chamber are all or nearly all normal.

15   Based upon the results of these measurements, a series
of parallel, rectangular chambers is established in which
the rate of fluid flow (see Figs. 4a and 4b over the lower
surface creates a shear stress rate which was found in the
tapered chamber to remove leukemic cells from the stroma
20   without removing all of the normal cells. In the case of
chronic myelogenous leukemia patient bone marrows, this
shear stress is typically 0.01-0.05 dynes/square cm. The
actual flow rate employed will depend on the size and
geometry of the chambers. Bone marrow cells from the
25   patient will be cultured in these rectangular chambers at a
concentration of 5×10^6/ml to 50×10^6/ml in Iscove's
Modified Dulbecco's Medium with 5-20% (typically 10%) Fetal
calf serum plus 0-15% (typically 10%) horse serum, with or
without 10^6M hydrocortisone. The bone marrow cells will be
30   cultured for 12-24 hours without fluid flow, and then fluid
flow will be initiated. The cells will be cultured for 3-7
days, at which time all of the nonadherent cells will be
discarded. The adherent cells will be recovered from the
rectangular plates by aspiration and mechanical agitation,

-41-

and then collected. These cells can then be either
directly returned to the patient, or stored in liquid
nitrogen by standard techniques for later use.

Cells other than those of the hematopoietic system
5   also may be separated using differential tolerance to shear
stress. Thus, where there are distinct subpopulations of
cells within a complex mixture of cells the methods
described above can be used to separate out a cell type of
interest from within a suspension of cells derived from,
10   e.g. skin, liver, muscle, nerve, or epithelium. Of
particular interest is the separation of tumor cells from
within a population of normal cells. The population of
cells to be separated will be contacted with a suitable
stromal substrate as described below, such as a purified
15   protein or cellular component to which the cells of
interest adhere. The shear stress tolerance for each of
the adherent subpopulations is determined as described
above. The fluid flow can then be adjusted appropriately
so as to retain the desired subpopulation of cells on the
20   stroma. The desired cells are then collected as described
above.

A variety of packings may be used in the reactor to
provide for adherent growth of the cells, while maintaining
some physical separation between the stromal cells and the
25   hematopoietic cells, and while allowing for some contact or
close juxtaposition between the stromal cells and the
hematopoietic cells. In this way, the factors secreted by
the stromal cells may be readily taken up by the
hematopoietic cells to encourage their proliferation and,
30   as appropriate, differentiation and maturation.

The protein matrix to support the cells may take the
form of shredded collagen particles, e.g., sponges or

porous collagen beads, sponges or beads composed of
extra-cellular bone matrix protein from bone marrow, or
protein coated membranes, where the protein may be
collagen, fibronectin, hemonectin, RGDS peptide, mixed bone

5 marrow matrix protein, or the like. Pore sizes of
membranes will generally range from about 1 to 5μ to allow
for interaction between the different cell types, while
still retaining physical separation.

Membranes may be employed, which will be protein

10 coated. Various membrane materials may be employed such as
polypropylene, polyethylene, polycarbonate, polysulfonate,
etc. Various proteins may be employed, particularly
collagen or the other proteins which were indicated
previously. The membrane should have sufficiently small

15 pores, that the transformed cells may not pass through the
membranes, but may grow and form a confluent layer on one
side of the membrane and extend portions of the cell
membrane into the pores. Generally the pores will be in
the range of about 1 to 5μ. In this manner, the

20 hematopoietic stem cells may grow on the opposite side of
the membrane and interact with the transformed cells,
whereby factors may be transferred directly from the
transformed cells to the hematopoietic progenitor cells.
The progenitor cells, the stem cells, are able to attach to

25 the intruded cytoplasmic projections which have passed into
the pores. Hematopoietic differentiation from the stem
cells occurs on one side of the membrane and differentiated
progeny are unable to squeeze back through the pores, which
are already largely occupied by the stromal cell layer when

30 confluence is approached or reached. For example, one could
provide for a plurality of chambers in which stromal cells
may grow and the hematopoietic cells may be moved in
accordance with the chamber which has the stromal cells at
a subconfluent level. Thus, by having a movable barrier

-43-

between the chambers, when the stromal cells approach
confluence, generally after about 8-12 weeks, one could
open or remove the barrier between the chambers and allow
for the stromal cells to migrate into the new chamber and
5   allow for the hematopoietic cells to come in contact with
the subconfluent stromal cells, while the subconfluent
stromal cells feed the factors to the chamber comprising
the hematopoietic cells (Fig. 5a and Fig. 5b). The
transfer of the hematopoietic cells can be achieved by
10   appropriate flow rates or by other convenient means. One
can provide for various wells in the chamber, which are
divided by appropriate walls, after seeding in one well,
when the cells become confluent, cells will then move over
into the next well and seed the next well in a subconfluent
15   manner. Another modification of the system is one in
which, after 8-12 weeks in culture, the hematopoietic cells
are exposed to new, proliferating stromal cells. This is
accomplished in one of several ways. This exposure to
proliferating stromal cells is accomplished in one of
20   several ways. In the first technique, the culture are
exposed to EDTA for 3-5 minutes, which removes the
hematopoietic stem cells from the stromal cells. The
removed cells are then transferred to a new culture vessel,
which may itself contain bone marrow stromal cells seeded
25   3-7 days prior. This process is repeated every 8-12 weeks.
Another alternative approach is to add additional surface
area by increasing the volume of the cultures and adding
additional collagen beads to the cultures at 8-12 weeks.
Finally, small organic molecules or proteins, particularly
30   hormones, such as platelet-derived growth factor (at
100-500 ng/ml), interleukin 1 alpha, tumor necrosis factor
alpha, or basic fibroblast growth factor or other molecules
mitogenic to fibroblasts, can be added to the cultures
every 3-7 days. This exposure to stromal mitogenic
35   stimulatory factors promotes the continued proliferation of

-44-

bone marrow stromal cells, and their continued production
of hematopoietic growth factors. Thus, one can provide for
the continuous subconfluent stage of the stromal cells.

5   Continuous fluid flow can also be used to selectively
separate normal from cancerous cells within a bone marrow
population. In this approach, a radial flow chamber is
first used to determine the specific stromal adhesive
properties of normal versus cancerous cells, and then a
rectangular flow chamber with flow rates established to
10   achieve a shear stress sufficient to remove the cancerous
cells is used to preoperatively separate the normal and
cancerous cells.

The subject method and apparatus also provides for the
opportunity to recycle stem cells which are lost by the
15   flow of the perfusion medium. The surface membrane protein
marker CD34 substantially separates immature hematopoietic
cells from mature hematopoietic cells. Thus, by capturing
and recycling those cells which are CD34+, one may avoid
the loss of stem cells to the medium.

20   Various techniques may be employed for capturing and
returning the immature fraction of cells to the reactor.
For example, one could label the cells with an antibody
specific for CD34 and then use antibodies to the antibody
for collecting the CD34+ cells and recycling them to the
25   reactor. Alternatively to positive selection, one may use
negative selection, whereby one would remove the mature
cells employing antibodies to various markers associated
with mature cells, such as antibodies to glycophorin A,
CD33, MO1, OKT3, OKT4, OKT8, OKT11, OKT16, OKM1, OKM5 Leu7,
30   Leu9, Leu M1, Leu M3, and the like. Various antibodies are
available for markers specific for mature cells of the
various hematopoietic lineages, lymphoid, myeloid and

-45-

erythroid, and these antibodies may be used to remove the
mature cells from the effluent from the reactor, followed
by harvesting of the remaining cells and restoring them to
the reactor. In this way, one can avoid forced decline in
5 the cultures due to loss of stem cells and maintain
unlimited stem survival *in vitro*.

        Separation using antibody markers can be achieved in
various ways, using standard techniques, individually or in
combination, such as panning, fluorescence activated cell
10 sorting, antibodies bound to various surfaces, e.g.
polystyrene surface, metal microspheres and magnets, and
the like. The antibodies are bound to a surface which
allows for separation between adherent and non-adherent
cells or the antibodies are labeled, directly or
15 indirectly, which permits selection between labeled and
unlabeled cells.

        By following the subject procedures greatly extended
periods of *in vitro* growth of hematopoietic cells may be
achieved, generally providing *ex vivo* human hematopoiesis
20 for at least six months in culture, with granulopoiesis
being supported for at least four months and erythropoiesis
for at least three months. In addition, hematopoietic
progenitor cells are continuously generated throughout the
culture resulting in net expansions of progenitor cells of
25 over 10-fold from input cells.

        In addition, by following the subject procedures
greatly increased rates of stem cell division are
supported, permitting the efficient insertion of
retrovirally transfected genetic material. Genes inserted
30 by the appropriate retroviral vector during an initial two
week infection period can be expressed in up to 10-30% of

-46-

all progenitor and precursor cells arising during
subsequent culture for over four months in culture. These
subject procedures thus support the successful transfer of
genetic material into a highly proliferative human
5   hematopoietic stem cell.

    Figure 1 is a schematic view of a perfusion chamber.
Reactor 10 with cover plate 12 and floor plate 14 are
joined by bolts 16, held in position by wing nuts 18.
Three bolts are employed, so as to avoid warping. The
10   chamber 20 has three sections, the middle section 22
containing the support matrix for the stromal cells, the
bed of stromal cells, and the bone marrow cells. The
central section 22 is separated from the top section 24 and
the bottom section 26 by membranes or mesh 28 and 30
15   respectively. Conveniently, polysulfonate membrane may be
employed or a stainless steel mesh, whose mesh size is
small enough so that cells are contained within the central
section of the chamber. The separating interphase may be
placed in the chamber using an inner cylinder 27 which is
20   sectioned to provide the separating membrane mechanical
support. The top section 24 and the bottom section 26 need
not be identical and will have tubing or membranes across
which liquid media and gases are exchanged. The gases are
exchanged across a hydrophobic, e.g., silicone, tube whose
25   length (and thereby gas/liquid contact area) may be varied
to allow for sufficient gas fluxes to support the needs of
the cell population that is metabolizing in the central
section. The media can be pumped or withdrawn directly
from the top or bottom sections through port 32 and may be
30   fed through delivery tube 34.

    If desired, the top and bottom sections may be
eliminated by using an external oxygenator. In this
situation, the separating membrane is held in place under

the glass cylinder 36 which fits into cylindrical groove
plates 12 and 14 and the area inside of the cylindrical
groove is indented to allow for good flow distribution
across the membrane. This geometry allows the fluid from
5   the finite number of inlet ports to mix and for radial
pressure to equilibrate, leading to a uniform liquid flow
across the separating membrane. This setup is suitable for
chambers which have relatively few cells, so that
oxygenation does not become limiting.

10   In Figure 2 is depicted a schematic representation of
the loop that connects the perfusion chamber to the side
media reservoir, oxygenator, sensor chamber, and
sample/injection ports.

An external fresh media source 50 is pumped by means
15   of pump 52 to a media reservoir through line 56 and spent
media is withdrawn through line 58 from reservoir 54 by
means of pump 52 to the spent media container 60 for
further processing. A second pump 62 pumps media from the
media reservoir 52 through line 64 through a hollow fiber
20   oxygenator 66. The media is directed through line 68 to
the first chamber of bioreactor 70. As appropriate, a
means for injection of media component 82 is provided, for
introducing the component into line 68 for transport by the
media into the first chamber of bioreactor 70. The
25   component may be test components, additional factors, or
the like. The media from bioreactor 70 is directed through
central chamber 72 into the second chamber 74 of the
bioreactor. From there the media is directed by line 76 to
in-line sensors 78 for detecting the change in composition
30   of the media.

For example, it is desirable that the

glutamine:glucose ratio be in the range of about 1:5-8,
depending on the cell lines used; for instance, preferably
1:8 for transfected 3T3 cells. Furthermore, ammonium
concentrations will preferably be below about 2.0 mM and
5   lactate concentrations are preferably less than about 40
mM. By monitoring the effluent from the bioreactor, the
media introduced into the bioreactor may be modified,
oxygen partial pressure may be changed, gas flow rate may
be altered, various components may be augmented, or the
10   rate of perfusion may be slowed or increased.

From the sensors 78, the media is directed through
line 80 by means of pump 62 to the reservoir 54.

By means of the flow path described above, the media
in the side reservoir is slowly exchanged using a separate
15   pump. This organization allows for separate control of the
media exchange rate (the outer pump) and the flow rate
through the oxygenator and perfusion chamber. The former
is used to control the longer term change in the media
composition and perfusion, while the latter may be used to
20   control the dissolved oxygen tension and flow patterns in
the chamber. The use of a small mesh biocompatible
membrane allows for plug (piston) flow in the chamber and
thus allows the precise control of delivery of growth
factors and other special compounds that one may wish to
25   introduce to the hematopoietic cells and stromal cells in
very precise amounts.

After autoclaving the chamber and components of the
loop, the reactor is assembled in a sterile environment.
The media may be circulated through the side loop and
30   chamber for a few days while signs of contamination are
monitored. If sterile assembly is accomplished, the

-49-

central section of the chamber is inoculated with either
the extra-cellular matrix alone or a pre-inoculated
extra-cellular matrix support that contains the stromal
cells. The stromal cells are then either: 1) kept in the
5 chamber for a period of a few days while their metabolic
performance and/or growth factor responsiveness is
monitored and if results are satisfactory, the bone marrow
is inoculated; or 2) immediately seeded with bone marrow.
In either case, the cell layer is kept at the bottom of the
10 central section of the perfusion chamber. The cells lay
down additional extra-cellular matrix and the cell layer
adheres to the separating membrane. At this time, the
chamber may be inverted and the cell layer may then be
located at the ceiling of the central section. In this
15 configuration, the maturing cells will settle on the bottom
of the central chamber as they lose their adherence to the
stromal layer. This feature is important to prevent the
damage caused by mature cells to the stromal layer and/or
the less mature hematopoietic cells. This feature also
20 makes the continuous removal of mature cells easier.

These cells are harvested by withdrawing the cells by
syringe, or by continuously allowing the cells to flow out
of the chamber, by the pressure of the perfused medium,
through the exit tubing.

25 The stromal cells will, for the most part, be
fibroblasts transformed with one or more genes providing
for desired hematopoietic growth factors. The same or
different cells may be transfected with the genes,
depending upon the particular selection of host cells, the
30 same or different cells may be used for a plurality of
genes.

A wide variety of normal cells or stable lines may be

employed. However, it is found that not all cell strains
are permissible, since transformation of some cell lines
may result in the overgrowth of the cells. Desirably, the
cells which are employed will not be neoplastic, but rather
5   require adherence to a support. The mammalian cells need
not be human, nor even primate. A variety of nontransformed
cells may be included in the adherent cell layer as well,
including normal human bone marrow adherent cells, normal
human spleen adherent cells, and normal human thymic
10   epithelium.

Methods for transforming mammalian cells, including
fibroblasts, are well known and there is an extensive
literature of which only a few references have been
previously given. The constructs may employ the naturally
15   occurring transcriptional initiation regulatory region,
comprising the promoter and, as appropriate the enhancer,
or a different transcriptional initiation region may be
involved, which may be inducible or constitutive.

A large number of transcriptional initiation regions
20   are available which are inducible or constitutive, may be
associated with a naturally occurring enhancer, or an
enhancer may be provided, may be induced only in a
particular cell type, or may be functional in a plurality
or all cell types. The transcriptional initiation region
25   may be derived from a virus, a naturally occurring gene,
may be synthesized, or combinations thereof.

Promoters which are available and have found use
include the chromosomal promoters, such as the mouse or
human metallothionein-I or II promoters, actin promoter,
30   etc., or viral promoters, such as SV40 early gene
promoters, CMV promoter, adenovirus promoters, promoters
associated with LTRs of retroviruses, etc. These promoters

-51-

are available and may be readily inserted into appropriate
vectors which comprise polylinkers for insertion of the
transcriptional initiation region as well as the gene of
interest. In other instances, expression vectors are
5   available which provide for a polylinker between a
transcriptional initiation region and a transcriptional
termination region, also providing for the various signals
associated with the processing of the messenger for
translation, i.e., the cap site and the polyadenylation
10   signal. The construction of the expression cassette
comprising the regulatory regions and the structural gene
may employ one or more of restriction enzymes, adaptors,
polylinkers, *in vitro* mutagenesis, primer repair,
resection, or the like.

15   The expression cassette will usually be part of a
vector which will include a marker and one or more
replication systems. The marker will allow for detection
and/or selection of cells into which the expression
cassette and marker have been introduced. Various markers
20   may be employed, particularly markers which provide for
resistance to a toxin, particularly an antibiotic.
Preferably, gentamicin resistance is employed, which
provides resistance to G418 for a mammalian cell host. The
replication systems may comprise a prokaryotic replication
25   system, which will allow for cloning during the various
stages of bringing together the individual components of
the expression cassette. The other replication system may
be used for maintenance of an episomal element in the host
cell, although for the most part the replication system
30   will be selected so as to allow for integration of the
expression cassette into a chromosome of the host.

          The introduction of the expression cassette into the
host may employ any of the commonly employed techniques,

-52-

including transformation with calcium precipitated DNA, transfection, infection, electroporation, ballistic particles, or the like. Once the host cells have been transformed, they may be amplified in an appropriate

5 nutrient medium having a selective agent, to select for those cells which comprise the marker. Surviving cells may then be amplified and used.

Host cells which may be employed include African green monkey cell line CV1, mouse cells NIH-3T3, normal human

10 bone marrow fibroblasts, human spleen fibroblasts, normal mouse bone marrow fibroblasts, and normal mouse spleen fibroblasts. It should be noted that in some instances, depending upon the choice of vector and cell line, the cells may become neoplastic. It is important that the

15 resulting transformed cells be capable of adherence, whereby the transformed cells maintain binding to a support, such as protein sponges, protein coated membranes, or the like.

Once the vector for expressing the appropriate growth

20 factors has been constructed, it may be used to transform the cells by any convenient means. The resulting transformed cells may then be used to seed the supports, which have already been described. These supports may be introduced into the reactor or may be present at the time

25 of seeding in the reactor. The cells will be allowed to grow for sufficient time to ensure that the cells are viable and are capable of producing the desired growth factors.

The reactor may then be seeded as appropriate with the

30 hematopoietic cells. The hematopoietic cells may include substantially pure stem cells, a mixture of hematopoietic cells substantially free of mature hematopoietic cells of

-53-

one or more lineages, or a mixture comprising all or
substantially all of thevarious lineages of the
hematopoietic system, at various stages of their
maturation.

5   The cells are allowed to grow with substantially
continuous perfusion through the reactor and monitoring of
the various nutrients and factors involved. For the most
part, the primary factors will be provided by the stromal
cells, so that a steady state concentration of growth
10   factors will normally be achieved. Since conditioned
supernatants are found to be effective in the growth of the
hematopoietic cells, one can provide for a ratio of stromal
cells to hematopoietic cells which will maintain the growth
factor at a appropriate concentration level in the reactor.

15   Transfected stroma can provide for the introduction of
genes into human stem cells. In mice, retroviral mediated
gene transfer into stem cells is made possible by
pretreating mice with 5-FU and then growing the harvested
bone marrow cells in WEHI conditioned media, which contains
20   IL-3 and GM-CSF (Lemischka Cell (1986) 45:917). The
artificial stroma, grown with a retroviral packaging cell
line secreting a retroviral vector of interest, may be used
to efficiently introduce genes into human stem cells. For
example, human T-cells could be made resistant to HIV
25   infection by infecting stem cells with the retroviral
vector containing an HIV antisense sequence under control
of a CDC2 regulatory sequence (Greaves, Cell (1989)
56:979-986) which would allow for tissue specific
expression in T-cells. There would be a factor provided by
30   the retroviral packaging cell line essential for
replication of the retrovirus; this factor would be absent
in the hematopoietic target cells. Once the virus was
transferred to the hematopoietic target cells, it would no

-54-

able to replicate.

In Figs. 3a and b are depicted radial flow chamber 100
having inlet 102 and outlet 104, and with 25 chamber 106
where the arrows 108 indicate the direction of flow.
5   Hematopoietic cells 110 are seeded onto a stromal layer 112
in the chamber and grown. The flow rate will determine
which cells are able to adhere, the non-adherent cells 114
passing out through outlet 104.

In Figs. 4a and 4b, growth chamber 120 is provided
10   having inlet 122 and outlet 124. In Figure 4b, inlet 122
comprises a manifold 128 which feeds individual chambers
126 containing cells 110 and stroma 112 in the chamber 126
for growth and separation.

In Figures 5a and 5b are shown growth chambers in
15   which barriers 134, 136, 138 are removed schematically
during culture: barriers 134 at about week 8-10; barrier
136 at about week 18-20 and barrier 138, at about week
28-32.

Having generally described this invention, a further
20   understanding can be obtained by reference to certain
specific examples which are provided herein for purposes of
illustration only and are not intended to be limiting
unless otherwise specified.

<u>ILLUSTRATIVE CELL SEPARATION AND STAINING PROCEDURES</u>
25   <u>Separation of Bone Marrow Cells on Ficoll:</u>
     1.   Dilute the bone marrow sample at a 1:4 ratio in I-
MDM kept at room temperature (Iscove's Modified Dulbecco
Medium; GIBCO; Cat. No. 430-2200).

     2.   Carefully, layer 35 ml of the diluted bone marrow

-55-

sample onto 15 ml of Ficoll-Paque at room temp. (Sp. Gr.
1.077 g/cc; Pharmacia; Cat. No. 17-0840-02) in 50 ml
centrifuge tube.

5   3.   Centrifuge at 700 x g (1800 rpm on Beckman) for 30
min. at room temperature (20°C).

        4.   After centrifugation remove most of the upper
layer (leaving about 5 ml above interphase), collect the
interphase layer (bone marrow cells), and wash 3 times in
ice cold I-MDM according to the following:
10   First wash: 1400 rpm/15 min/4°C.
            Second wash: 1200 rpm/10 min/4°C.
            Third wash: 1200 rpm/10 rpm/4°C.

        5.   After the third wash the cells are suspended
either in media or a balanced salt solution etc...
15   (depending on whtat is to be done with them), and counted
after making a 1:10 dilution of the cells in acetic acid
(10 μl cell suspension = 90 μl of 2% acetic acid in PBS;
this will allow for counting WBCs only, since RBCs get
lysed in the acetic acid).

20   6.   Cells are then suspended to the desired final
concentration in the appropriate medium (for media refer to
the different applications).

<u>Fluorescent Staining for MY-10 Positive Bone Marrow Cells:</u>
Reagents
25   Standard Buffer:
            Powdered Bacto Dried DIFCO Buffer (Baxter; Cat. No.
            2314-15GB):   200 g

            10% NaN$_3$ (sodium azide):   20 g

            Heat inactivated fetal calf serum (56°C, 30 min.)200 ml

-56-

Bring volume to 20 liters in dd-H$_2$O; pH 7.15-7.25;
store at 4°C

Good for 1 month

2% Paraformaldehyde solution

5   paraformaldehyde: 10 g

           dd-H$_2$O: 500 ml

           10N NaOH (under the hood)   8-20 drops

           powdered Bacto dried Difco Buffer   5g

           Pour dd-H$_2$O in 500 ml flask and stir on a hot plate to
10   60°C in the hood.

           Add 10 gr. parafromaldehyde

           Add NaOH dropwise until solution clears

           Add 5 gr DIFCO

           Let cool, Adjust pH to 7.35-7.45 with 2 N HCl.

15   1. After counting the cells, they are washed once in
standard buffer (100 rpm, 5 min. 4°C).

           2. Cells are then suspended in standard buffer at the
concentration of 2 x 10^5 cells/ml.

           3. Two 50 μl aliquots of the cells are deposited into
20   2 15 ml centrifuge tubes.

           4. To one tube 50 μl of a 1:5 dilution of anti-HPCA-1
are added (anti-human progenitor cell antigen; Becton
Dickinson; Cat. No. 7660; diluted 1:5 in standard buffer).
To the other tube, 50 μl of a 1:5 dilution of MIg are added
25   (Mouse IgG1 control; Becton Dickinson; Cat. No. 9040;
diluted 1:5 in standard buffer).

-57-

5. Both tubes are then incubated for 1/2 hour on ice.

6. After incubation; cells are washed twice in 5 ml standard buffer (1000 rpm, 5 min, 4°C).

7. After the second wash, cell pellets are
5   resuspended in 50 µl of a 1:40 dilution of GAM-FITC
     (Affinity isolated Goat F(ab')2 anti-mouse IgG and IgM,
     human Ig adsorbed, Fluorescein conjugated; TAGO; Cat. No.
     4353; diluted 1:40 in standard buffer).

8. Cells are incubated for 1/2 and hour in the dark,
10   on ice.

9. After incubation, cells are washed twice in 5 ml
standard buffer (100 rpm, 5 min., 4°C), and each of the
pellets is resuspended in 100 µ standard buffer plus 100 µl
2% paraformaldehyde solution.

15   10. Cells are then analyzed for fluorescence using
the flow cytometer. Percent positive fluorescence
constitute % fluorescence in anti-HPCA-1 sample minus %
fluorescence in MIg sample.

Fluorescent Staining of Bone Marrow Cells to Sort Out
20 Mature Progenitor Cells:
Objectives:
     The purpose of this staining is to enrich for
hematopoietic stem cells (most primitve stem cells) by
removing mature cell populations, using the flow cytometer
25 or magnetic beads. It is always a good idea to retain some
cells as total bone marrow cells (after Ficoll separation)
to stain for MY-10 positive cells in order to compare with
the outcome of sorting and determine extent of enrichment.

-58-

1.   Cells are separated on Ficoll-Paque as described
before.   (Remove 0.5 x 10^6 cells and divide into 2 portions
to stain with anti-HPCA-1/GAM-FITC and MIg/GAM-FITC).

5   2.   After the third wash the cell pellet is suspended
in the monoclonal antibody cocktail (refer to section
describing the making of this cocktail) using 1 ml of the
cocktail per 10^7 cells, and cells are incubated on ice for
1 hour.

3.   Cells are then washed three times in excess ice
10   cold I-MDM (1000 rpm, 5 min, 4°C).

4.   After the third wash cells are suspended in a 1:40
dilution of GAM-FITC (diluted in I-MDM, not standard
buffer) at the rate of 50 μl per 0.25 x 10^6 cells, and
incubated on ice in the dark for 1/2 an hour.

15   5.   After incubation, cells are washed three times in
ice cold I-MDM, and after the final wash they are suspended
in 2-4 ml of ice cold I-MDM and kept on ice until sorting.

6.   Cells are then sorted on the flow cytometer based
on fluorescence, to eclude the upper 85% of the
20   fluorescence histogram.   Sorting could be repeated twice
for better enrichment.

7.   After sorting, cells are counted, washed, and an
aliquot is stained for MY-10 positive cells (as described
above) to determine the extent of enrichment in comparison
25   to stained aliquots of the total bone marrow cells.

Selecting for Immature Cells by Using Magnetic Antibodies:
1.   Follow steps 1-3 in the procedure for Fluorescent
staining of bone marrow cells to sort out mature cells.

Note: sodium azide is not included in any of the buffers.

2.   In the meantime wash an appropriate amount of
magnetic goat anti-mouse Ig (Biomag; Collaborative
Research; Cat. No. 74340-50; 1 mg/ml; 5 x 10^8 particles/ml)
5   3 times in ice cold I-MDM at 1500 rpm, 5 min. 4°C (to wash
off the sodium azide which is used as a preservative).

3.   Resuspend the cell pellet obtained after the third
wash in "step 1" in Biomag at the rate of 50 particles
Biomag/cell (e.g., for 1 x 10^6 cells use 5 x 10^7 particles,
10   therefore, 0.1 ml of Biomag).

4.   Deposit the cells in a T-25 or T-75 tissue culture
flask (depending on cell numbers) and incubate on ice for
1/2 hour with intermittent shaking.

5.   After incubation, lay the flask onto the flat
15   magnet (provided with the Biomag), secure with a rubber
band or tape, and incubate at 4°C for 10-15 min.

6.   Stand the magnet and the flask into an upright
position and collect the supernatent.

7.   Repeat steps 4-6 two more times.

20   8.   Count the cells, wash once in ice cold I-MDM,
remove an aliquot to stain for MY-10 positive, and
resuspend in appropriate media for further use.

-60-

I.   Medium Replacement

Materials and Methods:

    Cells: Human bone marrow cells were obtained from
heparinized aspirates from the iliac crest of informed and
5   consenting individuals. The bone marrow was separated by a
Ficoll-Paque (Pharmacia, No. 17-0840-02) density gradient
centrifugation and the low density cells (<1.077 gm/cm^3)
were collected and washed 3 times with Iscove's Modified
Dulbecco's Medium (IMDM). The cells were counted between
10   the second and third washes. The cells were then seeded
onto 24-well tissue culture plates (Costar No. 3524) in
duplicate or triplicate at 1, 2, and $5 \cdot 10^6$ cells/ml at 322
μl/well.

    Long-term culture conditions: The low density cells
15   were incubated in IMDM supplemented with 10% fetal calf
serum (Hyclone Laboratories, 10% horse serum (Hyclone
Laboratories), 1% penicillin/streptomycin (Sigma, 10,000
U/ml penicillin G and 10 mg/ml streptomycin, Cat. No.
P3539), and 10^{-5} M hydrocortisone (17-Hydroxy-
20   corticosterone, Sigma, Cat. No. H0888) in a humidified 5%
CO_2/95% air atmosphere. The cultures were treated with one
of three medium exchange schedules, 100% daily medium
exchange (7/wk), 50% daily medium exchange (3.5/wk), or 50%
biweekly medium exchange (1/wk). Twice per week during the
25   medium exchange, 50% of the non-adherent cells were removed
from each culture well and counted using a hemocytometer.

    When the cells were removed for counting (twice/week),
all of the medium removed during feeding of the 3.5/wk and
1/wk cultures was saved for cell counts and fresh medium
30   returned to the wells. The 7/wk cultures required saving
1/2 of the removed medium for cell counts, while
centrifuging and returning the non-adherent cells in the

remaining 1/2 of the medium removed. Fresh medium was then
added to each well to replace the medium removed for cell
counts. On days when the cells were not removed for
counting, 100% or 50% of the medium was removed from each
5   of the 7/wk and 3.5/wk culture wells respectively, the
cells were centrifuged and returned to the original wells
with additional fresh medium.

Methylcellulose and morphologic assays: One every
other week the non-adherent cells removed for cell counts
10   were plated in methylcellulose in the presence of
erythropoietin, GM-CSF, and IL-3, and the Granulocyte
Macrophase-Colony Forming Units (CFU-GM) were enumerated.
Aliquot of removed cells were cytocentrifuged, stained with
Wright-Giemsa, and differential cell counts performed.

15   Statistical analysis: The biweekly cell production
results are expressed as the mean ±SEM from replicate
cultures. The probability of significant differences
between groups of cultures was determined by comparing the
normalized cumulative cell production values from the
20   rapidly exchanged cultures (7/wk and 3.5/wk) to the matched
control cultures (1/wk) using a paired t-test. Statistical
significance was taken at the 5% level.

Results:
      Kinetics of nonadherent cell production: Nonadherent
25   cell production was examined both as a function of inoculum
cell density (over the range $1-5 \cdot 10^6$ cells/ml) and medium
exchange rate. The medium exchange rate was varied from
one medium volume exchange per week, the traditional Dexter
culture rate, to seven medium volume exchanges per week.
30   The biweekly number of cells collected was normalized by
dividing by the number of cell inoculated per culture.

At each medium exchange rate, the normalized cell
collection curves did not change significantly with
inoculum density. The cell production for the cultures
maintained at the three medium perfusion rates of 7/wk,

5   3.5/wk and 1/wk were similar when normalized to the number
of cells inoculated per culture. Comparison of the final
cumulative cell productions between inoculum densities
showed no significant differences, at any of the three
medium exchange rates ($p > .20$ by a paired t-test for all

10   pairs of samples).

The medium exchange rate, in contrast, strongly
influenced the rate and longevity of cell production in
these cultures. Cell production of the cultures exchanged
at 1/wk (control), 3.5/wk, and 7/wk all decayed over the

15   first few weeks. Differences in culture productivity,
however, became apparent after week 3 in culture. Between
weeks 3 to 10, the cell production was constant in the 7/wk
cultures, constant at a lower level in the 1/wk cultures,
but increased exponentially in the 3.5/wk cultures. After

20   weeks 10 to 12, cell production declined in all cultures
until culture termination.

Results for the 1/wk exchanged cultures are equivalent
to those commonly observed in traditional human Dexter
cultures in a variety of systems, whereas the rapidly

25   exchanged cultures of 3.5 and 7/wk showed increased cell
productivity when compared to previous optimum culture
methods. Cultures in which 1/2 of the medium was exchanged
daily (3.5/wk) maintained increased cell production for
substantially longed than either the control (1/wk) or

30   complete daily exchange (7/wk) cultures. Between weeks 3
and 9, the number of nonadherent cells collected from the
3.5/wk exchanged cultures increased exponentially with a
doubling every 2.1 weeks.

The cell production under the 3.5/wk and 1/wk
protocols can be directly compared by plotting the cell
production under the 3.5/wk exchange rate as a percentage
of the production of the cultures with an exchange rate of

5   1/wk. This comparison shows that during the initial decay
phase the cell production under the two protocols is
similar. However, between weeks 3.5 and 18, the cell
production under the 3.5/wk exchange rate is consistently
higher.

10   The proliferative potential of the cultures can thus
be measured by their ability to produce cells following the
initial decay. The normalized cumulative cell production
following week 3 ($\Sigma_{i=7}^{n}$, C_i/C_o was independent of the cell
inoculation density for the medium exchange rates of 7/wk,

15   3.5/wk. Cell production data from the cultures at similar
medium exchange rates were qualitatively and statistically
similar, and were therefore density averaged and combined
(bottom panel) to obtain a larger statistical sample. The
density averaged cumulative cell production between weeks

20   3.5 and 20 was: 0.22 for the 7/wk; 0.40 for the 3.5/wk;
and 0.15 for the 1/wk cultures. The increase in the medium
exchange rate from 1/wk to 7/wk thus increased the cell
production about 60% over the typical Dexter culture medium
exchange schedule. The 3.5/wk exchange rate resulted in

25   almost 3-fold cumulative cell production increase compared
to the 1/wk Dexter protocol. Statistical analysis of these
data using a paired t-test, demonstrated significant
differences between both the 7/wk vs. 1/wk and the 3.5/wk
vs. 1/wk at the 5% level of significance. The medium

30   exchange rate of 3.5/wk thus improves the cell production
rate over the traditional Dexter protocol of 1/wk.

<u>Granulocyte-macrophase progenitor cell production</u>:
Granulocyte-macrophase progenitor cell assays were

-64-

performed from replicates of a given medium perfusion
schedule and inoculum density (Table 2). The medium
perfusion rate had a pronounced effect on the number of
granulocyte-macrophage progenitor cells produced. The
5   3.5/wk medium exchange cultures showed the greatest
longevity in terms of progenitor cell production. These
cultures produced progenitors at a stable rate between
weeks 4 and 18.

The optimum conditions in terms of progenitor cell
10 production are the cultures exchanged 3.5 times per week
and inoculated at $5 \cdot 10^6$ cells/ml. These cultures
produced a significant number of progenitor cells until
week 20. Statistical analysis, using a paired t-test,
showed that the optimum medium exchange rate cultures of
15 3.5/wk produced significantly more granulocyte-macrophage
progenitor cells after week 8 than did the corresponding
7/wk and 1/wk cultures at all three inoculation densities
at the 1% level of significance. The number of progenitor
cells produced is important as it is an indirect measure of
20 stem cell renewal. Progenitor cells can only be present
after several weeks in culture by differentiation from an
earlier cell, presumably a stem cell, which is still
present in culture. Thus, these data suggest that more
physiologic, rapid medium/serum exchange rate and higher
25 cell densities may have provided conditions that supported
some degree of stem cell renewal for five months.

Nonadherent cell morphology: To determine whether the
prolonged hematopoiesis supported by the 3.5/wk cultures
was qualitatively different from the other cultures, the
30 non-adherent cells collected between weeks 10 and 19 were
stained and typed morphologically. At the exchange rates
of 1/wk and 7/wk, the cells produced were mostly
macrophages by week 15 and thereafter (Table 3), which is

similar to results from studies in other laboratories.   In
contrast, the cultures perfused at a rate of 3.5 medium
volumes per week and seeded at 5 • 10^6 cells/ml produced
granulocytes as well as macrophages through week 19.   Thus,
5   it seems that this medium exchange rate and inoculum
density more effectively reconstituted granulopoiesis *in
vitro.*

Table 2: The average number of nonadherent progenitor cells removed from long term bone marrow cultures (LTBMCs) as a function of the medium perfusion rate and inoculum density.

Week	5x10^6 per ml	7/wk 2x10^6 per ml	1x10^6 per ml	5x10^6 per ml	3.5/wk 2x10^6 per ml	1x10^6 per ml	5x10^6 per ml	1/wk 2x10^6 per ml	1x10^6 per ml
2	237±27	11±3.3	106±5	120±16	132±7.9	167±13	368±29	94±20.8	335±46
4	149±21	101±5.1	104±10	93±10	37±5.6	20±0	21±1.3	2±0	8±4.4
6	47.7±7	12±2.5	8±0	17±3	6±4.1	5±2.7	13±5.1	1±0	1±0
8	40±3	0	4±0	38±6	24±2.7	10±3	34±7.4	0	0
10	0	0	0	28±8.3	10±2.9	5±1.3	8±2.3	2±2.3	0
12.5	0	6±2.3	0	8±2.3	0	0	0	0	0
14	0	0	0	22±6.4	6±1.3	2.5±1.2	3±1.3	0	0
16	6±2.2	0	0	24±7.6	4±1.7	2±1.3	9±3.6	0	0
18	0	0	0	24±6.3	4±1.3	0	0	0	0
20	0	0	0	5±0	4±0	3±0	1±0	0	0
22	2±1.3	0	0	4±1.3	10±3	0	0	0	0
10-22*	8±3.5	6±2.3	0	115±32.2	40±11.2	12.5±3.8	21±7.2	2±7	0

Replicate samples at each medium perfusion rate and inoculum density were pooled and are each tabulated as one mean ± SEM. Cumulative CFU-GM production after week 8 is statistically greater in the 3.5/wk cultures than the corresponding cultures perfused at 7/wk or 1/wk at all inoculum densities at the 1% level of significance.

-67-

This result supports the hypothesis that long-term human
Dexter culture conditions are suboptimal and as a culture
in vitro better approximate the hematopoietic environment
in vivo, more effective reconstitution of bone marrow *ex*
5   *vivo* can be attained.

Physical appearance: The medium exchange rate
significantly affected the physical appearance of the
cultures. By 10 weeks in culture, the 7/wk cultures had
large number of adipose cells in the stroma while the
10   3.5/wk cultures had few fat cells and the 1/wk cultures
never developed fat cells. At culture termination at 26
weeks, the stroma of the 7/wk cultures were composed of
approximately 20-30% fat cells while the 3.5/wk cultures
still only had a few fat cells. Adherent colony
15   distribution also varied between cultures with different
medium perfusion rate. Adherent colonies in the 3.5/wk
cultures persisted longer than those in the 7/wk and 1/wk
cultures.

Table 3: Nonadherent cell morphology as a function of the medium perfusion rate and inoculum density.

		5×10^6 per ml			2×10^6 per ml			1×10^6 per ml		
Medium perfusion rate	weeks	%Mø	%G	% myeloid pre-cursors	%Mø	%G	% myeloid pre-cursors	%Mø	%G	% myeloid pre-cursors
7/wk	10.4	25	57	18	57	32	11	52	34	14
	13.4	49	34	17	92	5	3	63	22	15
	15.4	66	19	16	79	19	2	54	17	29
	19	93	5	1	96	3	1	100	0	0
3.5/wk	10.4	50	27	23	45	38	17	39	45	17
	13.4	23	59	19	27	56	17	36	47	17
	15.4	41	38	21	44	27	29	67	13	21
	19	58	37	5	88	9	3	99	1	0
1/wk	10.4	59	21	20	60	11	29	ND	ND	ND
	13.4	56	25	20	19	36	46	43	7	50
	15.4	76	4	20	ND	ND	ND	46	39	15
	19	100	0	0	100	0	0	100	0	0

Data are for pooled replicate samples at each medium perfusion rate and inoculum density and are shown as the percentage of macrophages (%Mø), granulocytes (mature granulocytes and bands, %G), and immature granulocytes (metamyelocytes and less mature cells, % myeloid precursors).

II. Medium Replacement Combined with Supplementation of
            Medium with Hematopoietic Growth Factors

Materials and Methods:

Cells: Human bone marrow cells were obtained
5   following informed consent from heparinized aspirates of
the iliac crest bone marrow, under a protocol approved by
the University of Michigan Human Investigation Committee.
The bone marrow was separated by a Ficoll-Paque (Pharmacia)
density gradient centrifugation and the low density cells
10   (<1.077 gm/cm^3) were collected and washed 3 times with IMDM.
The cells were counted between the second and third washes.
The cells were then seeded onto 6-well tissue culture
plates (Costar No. 3406) or collagen coated 6-well plates
(rat tail type 1 collagen, Biocoat. Collaborative Research
15   Inc. Cat. No. 40400) in duplicate $5 \cdot 10^6$ cells/ml at 1.5
ml/well.

Culture medium: The medium used was IMDM (Gibco
Laboratories. Cat. No. 430-2200) containing 10% fetal calf
serum (Hyclone Laboratories), 10% horse serum (Hyclone Lab-
20   oratories), 1% penicillin/streptomycin (Sigma, 10,000 U/ml
penicillin G and 10 mg/ml streptomycin, Cat. No. P3539),
and 10^{-5}M hydrocortisone (17-Hydroxycorticosterone, Sigma,
Cat. No. H0888).

Hematopoietic growth factors (HGH): Due to the
25   frequent culture supplementation via rapid medium exchange,
hematopoietic growth factors were added to the medium at
approximately 1/20 of the concentrations found to promote
maximal colony formation in clonal assays 4. The
concentrations used were 1 ng/ml of IL-3, 1 ng/ml of GM-CSF
30   (Amgen Biologicals, Cat. No. 13050), 0.1 U/ml of Epo (Terry
Fox Labs. Vancouver, Canada).

Hematopoietic progenitor cell assay: Nonadherent

-70-

hematopoietic cells removed from culture were counted and
plated at $1 \cdot 10^5$ cells/ml or fewer cells in methylcellulose.
GM-CSF and Epo were added to the methylcellulose at 20
ng/ml and 2 U/ml, respectively. The cell were plated in 24
5   well plates at 0.25 ml/well and incubated at 37°C for 14
days. The colonies were then counted under an inverted
microscope and colonies greater than 50 cells were scored
as GM-colony forming units (CFU-GM), erythroid
burst-forming unit (BFU-E), or granulocyte erythroid
10   megakaryocyte macrophage-colony forming unit (CFU-GEMM).

     LTBMC conditions: The cultures were incubated at 37°C
in a humidified 5% CO_2/95% air atmosphere and perfused
(medium exchanged) at a rate of 50% daily medium exchange.
During the first week in culture, all cells removed during
15   the daily medium exchange were centrifuged and returned to
the original wells. After the first week in culture, 50%
of the total nonadherent cells were removed from the
cultures on a biweekly basis during the medium exchange,
mononucleated cells counted, and fresh medium returned to
20   the wells. The remaining five days per week when the cells
were not counted, 50% of the medium was removed from each
of the culture wells and replaced with fresh medium, the
removed medium was centrifuged, the medium decanted from
the cell pellet, and the cells returned to their original
25   wells.

     Statistical analysis: The probability of significant
differences between groups of cultures was determined by
comparing the normalized cumulative cell production values
from the rapidly perfused cultures supplemented with
30   hematopoietic growth factors to the matched untreated
control cultures using a paired t-test. Statistical
significance was taken at the 5% level. There were no
statistical differences between matched rapidly perfused

-71-

LTBMCs cultured on tissue culture plastic and type I rat
tail collagen at the 5% level. Therefore, the data for the
plastic and collagen matrix were combined for presentation
in this and all other figures and statistical analysis
5 performed on the combined data.

Results:
    Kinetics of cell production in rapidly exchanged
growth factor supplemented LTBMCs: As a first test of the
hypothesis that the longevity and productivity of long term
10 bone marrow cultures (LTBMCs) is limited by insufficient
production of HGF's, we maintained rapidly exchanged ex
vivo bone marrow cultures that were supplemented with IL-3,
or Epo. In these cultures, 50% of the medium was removed
daily and replaced with an equal volume of fresh medium
15 supplemented with IL-3 or Epo. The cells removed were then
centrifuged, the medium decanted and discarded, the cells
resuspended, and the cells returned to the original
cultures. IL-3 and Epo individually enhanced the cell
productivity of rapidly exchanged LTBMCs. The cultures
20 containing Epo alone initially had a high cell production
rate due to substantial terminal erythroid differentiation.
However, by week four erythropoiesis had ceased and the
cell production rate had decreased to the level of the
control cultures. IL-3 and Epo induced an average increase
25 in nonadherent cell production over controls throughout the
18 weeks of culture of 175% and 173%, respectively.

    Combinations of growth factors proved to be more
effective in increasing the nonadherent cell production
rate. The highest rate of cell production was observed for
30 the combination of IL-3+GM-CSF+Epo. These cultures
produced approximately 25% of the number of cells
inoculated biweekly during the first 6 weeks in culture and
had an average 4.8-fold increase in nonadherent cell

production over controls during weeks 2-8. The combination
of IL-3+GM-CSF produced an average 3.5-fold increase in
nonadherent cells as compared to controls through week 8.
In separate experiments, adding neither IL-6 nor G-CSF to

5   the combination of IL-3+GM-CSF+Epo improved the nonadherent
cell production rate, but instead resulted in cell
production rates indistinguishable from the cultures
containing the combination of IL-3+GM-CSF. In all cases,
the stimulatory effect on cell production induced by the

10   addition of HGFs was maximal between weeks 0 to 8, although
cell production was higher than the controls throughout the
culture.

The combinations of HGFs lead to high absolute numbers
of nonadherent cells produced in rapidly exchanged LTBMCS.

15   The productivity of the cultures can be shown by comparing
the cumulative number of cells produced over time ($\Sigma_{i=1}^{n}$, C_i,
C_i being the number of nonadherent cells collected at time
i), relative to the number of cells inoculated (C_o) by
plotting the ratio ($\Sigma_{i=1}^{n}$, C_i, C_o) as a function of time.

20   When this ratio exceeds unity, a culture has produced more
cells than were inoculated and the culture has led to an
expansion in cell number.

The combination of IL-3+GM-CSF+Epo induced cumulative
cell production that was more than 3-fold greater than the

25   number of cells inoculated. The cell production rate was
the highest during the first 6 weeks in culture during
which time the culture produced approximately as many cells
as were inoculated every two weeks. This maximum cell
production rate was 15% of the estimated *in vivo* bone

30   marrow cell production rate where 50% of the myeloid cell
mass is generated daily. The combination of IL-3+GM-CSF
resulted in more than a 2-fold expansion in cell number and
at rates comparable to the combination of IL3+GM-CSF+Epo
during weeks 3-7 in culture. Untreated rapidly exchanged

(50% daily medium exchange) and slowly exchanged (50%
medium exchange biweekly) control cultures not supplemented
with HGFs produced approximately 1 and 0.37 times the
number of cells inoculated after 18 weeks, respectively.

5   More importantly more than half of all cells removed from
these unsupplemented cultures came from the first two
samplings, indicating that many of these cells were from
the original inoculum and that supplementation of the
cultures with HGFs are required to induce significant

10   cycling of progenitor and stem cells.

     Morphologic analysis of nonadherent cells: The
addition of multiple HGFs also increased the variety of
myeloid cells produced in the cultures. The control
cultures produced nonadherent cells that were predominately

15   macrophages after week 3 in the culture. Production of
erythroid cells decreased rapidly with few erythroid cells
detected after week 5. The cultures containing Epo (Epo
alone, IL-3+Epo, and IL-3+GM-CSF+Epo) produced a transient
increase in erythroid cell production, with a high

20   percentage (55-75%) of nonadherent cells being erythroid
through week 3. When IL-3+Epo±GM-CSF was present, the
cultures continued to produce erythroid cells throughout
the 16 weeks in culture with about 5-15% of the nonadherent
cells being typed as erythroid. Thus, in the presence of

25   IL-3+Epo, erythropoiesis was active throughout.

     IL-3 ± Epo led to a nonadherent cell population that
was predominately (60-70%) late granulocytes (LG) at week
5. The percentage of LGs steadily declined until it
reached about 20% at week 18. The production of

30   macrophages rose correspondingly. When GM-CSF was added to
IL-3±Epo, the high percentage of LG persisted through 18
weeks. The combination of IL-3+GM-CSF thus led to active
granulopoiesis for 18 weeks in culture, and the addition of

Epo maintained erythropoiesis as well. Photomicrographs of
the control and IL-3+GM-CSF+Epo supplemented cultures at
5.5 weeks in culture show the dramatic enhancement in
culture density and variety of cells produced.

5   Kinetics of nonadherent progenitor cell production:
Progenitor cell production increased with the addition of
multiple HGFs. The production of granulocyte macrophage
colony forming units (CFU-GMs) in the untreated controls
was prolonged and steady for over 18 weeks, which is
10   consistent with the earlier results obtained using rapidly
perfused LTBMC without HGF. CFU-GM produced in the
IL-3+GM-CSF and IL-3+Epo±GM-CSF cultures was approximately
10-fold higher than controls during weeks 3 to 5.

       Erythroid burst forming unit (BFU-E) production in
15   human LTBMC has been reported to be low and cease quickly
(Coutinho et al, Blood (1990) 75(11): 2118-2129). The
rapidly exchanged, untreated controls exhibited a rapid
decrease in BFU-E production although low levels of BFU-E
were produced through 17 weeks in culture. The addition of
20   Epo alone did not significantly influence the number of
BFU-Es produced. IL-3 alone induced a mild short-lived
stimulation of BFU-E production in weeks 3-5. On the other
hand, IL-3 plus either Epo or GM-CSF induced a 10 to
20-fold elevation of nonadherent BFU-E levels compared to
25   that of controls during weeks 3 to 5 of culture.

       III.   Transformation of human stem cells
Materials and Methods
       Cells Human bone marrow cells were obtained following
informed consent from heparinized aspirates of the iliac
30   crest bone marrow, under a protocol approved by the
University of Michigan Human Investigation Committee. The

-75-

bone marrow was separated by a Ficoll-Paque (Pharmacia)
density gradient centrifugation and the low density cells
(<1.077 gm/rm^3) were collected and washed 3 times with
IMDM. The cells were counted between the second and third
5   washes. For the CD18 gene transfer experiments, bone
marrow was obtained following informed consent from a CD18
deficient patient donor.

Lineage negative (Lin⁻) selection of bone marrow cells
Mature mononuclear cells were removed from the above cell
10   preparation by incubating the cells with a mixture of
monoclonal antibodies (MAb) after the third wash with IMDM.
10^7 cells were incubated in 1ml of MAb cocktail on ice for
1 hour with gentle mixing every 10-15 minutes. The MAb
cocktail used is shown in Table 4.

-76-

Table 4:   Preparation of monoclonal antibodies used to
           separate lineage[+] bone marrow mononuclear cells.

Monoclonal Antibody	Specificity	Cat. No.	Conc. $\mu g/10^9$ cells	Volume (μl)
Anti-Leu-1	T-cell	6300	30	125
Anti-Leu-5b	T-cell, E-rosette receptor	7590	32	16
Anti-Leu-10 (Anti-HLA-DQ)	B-cells, monocytes	7450	250	125
Anti-Leu-12	B-cells	7540	120	60
Anti-CALLA	Common Acute Lymphoblastic Leukemia	7500	60	60
Anti-Leu-M1	Monocytes, granulocytes	7420	1000	500
Anti-MO-1	Macrophages	-	$1000\mu l$	1000
Anti-10F-7	RBCs	-	$2000\mu l$	2000
Anti-E3	RBCs	-	$2000\mu l$	2000

Add IMDM to make MAb cocktail total volume of 100 ml, filter sterilize,
aliquot in 1 ml columns, and store at -20°C.

The cells were washed 3 times in excess ice cold IMDM
and centrifuged at 4°C.   An appropriate amount of magnetic
goat anti-mouse Ig (Biomag; Collaborative Research Corp.,
Cat. No. 74340-50, 1mg/ml, $5x10^8$ particles/ml) was washed 3

5   times in ice cold IMDM and centrifuged.   The cells were
resuspended in Biomeg at 50 particles/cell and placed in a
T-25 or T-75 tissue culture flask and incubated on ice for
½ hour with intermittent shaking.   After incubation, the
flask was laid onto flat magnet, the magnet secured to the

10   flask and incubated at 4°C for 10-15 minutes.   The magnet
and flask were stood upright and the supernatant collected.
The incubation with shading, adding magnet, standing
upright, and collecting supernatant was repeated 2 more
times.   The cells were counted and seeded onto 6-well

15   tissue culture plates.   (Costar No. 3406).

-77-

Culture medium The medium used was IMDM (Gibco
Laboratories, Cat. No. 430-2200) containing 10% fetal calf
serum (Hyclone Laboratories), 10% horse serum (Hyclone
Laboratories), 1% penicillin/streptomycin (Sigma, 10,000
U/ml penicillin G and 10 mg/ml streptomycin, Cat. No.
P3539), and 10^{-5}M hydrocortisone (17-Hydroxycorticosterone,
Sigma, Cat. No. H0888).

Hematopoietic growth factors The hematopoietic growth
factors were the optimum discussed above. The
concentrations used were 1 ng/ml or 0.4 U/ml of IL-3 (a
gift from Genetics Institute, Cambridge, MA), 1 ng/ml of
GM-CSF (a gift from Genetics Institute, Cambridge, MA), 50
U/ml of IL-1a (Genzyme Corp.), 0.1 U/ml of Epo (Terry Fox
Labs, Vancouver Canada), 10 ng/ml MGF (mast cell growth
factor, the c-kit ligand, Immunex Corp., Seattle, WA), and
2.0 ng/ml Hybrikine ([PIXY321] Immunex Corp., Seattle, WA).

Hematopoietic progenitor cell assay Nonadherent
hematopoietic cells removed from culture during the weekly
sampling were counted and plated at $1-10^5$ cells/ml or fewer
cells in methylcellulose. MGF, GM-CSF and Epo were added
to the methylcellulose at 50 ng/ml, 20 ng/ml, and 2 U/ml,
respectively. The cells were plated in 24 well plates at
0.25 ml/well and incubated at 37°C for 14 days. The
colonies were then counted under an inverted microscope and
colonies greater than 50 cells were scored as a GM-colony
forming units (CFU-GM), erythroid burst-forming unit
(BFU-E), or granulocyte erythroid megakaryocyte macrophage-
colony forming unit (CFU-GEMM).

Retroviral producer cell lines Two retroviral producer
cell lines were obtained from Dr. Eli Gilboa's Laboratory
at Memorial Sloan Kettering Cancer Center, New York, NY.
The cell line produce amphotrophic viral particles that

contain the NEO gene which produces neomycin
phosphotransferase providing resistance to the mammalian
neomycin analog G418.   Also both cell lines produce
retroviral particles that are deficient in the required
5   retroviral genes so that cells infected with the retrovirus
cannot themselves produce infectious virus.

The SAX containing packaging cell line is a 3T3 based
cell line which contains a modified Moloney Murine Leukemia
Virus (MoMuLV).   The SAX provirus contains the NEO gene, an
10   SV40 promoted Adenosine deaminase gene, in a XhoI
restriction site.   Also, the SAX provirus contains the ψ
packaging region but is deficient in the gag (core
proteins), pol (reverse transcriptase), and env (envelope
proteins) genes.   This second retroviral particle contains
15   a double copy of the foreign DNA and the retroviral
particles are denoted DC-29 (double copy-29th clone).   The
DC-29 provirus contains two copies of the NEO gene and
other retroviral and foreign DNA in a 3T3 cell line.

For the CD18 experiments, the amphotropic packaging
20   cell Psi-Crip infected with a retroviral vector containing
a human full length CD19 cDNA was used (Wilson et al,
Science (1990) 248:1413-1416).   In this retrovirus, a full
length cDNA for human CD18 was cloned into the BamH1 site
of a vector that expresses the recombinant gene from
25   heterologous sequences spanning the 5' region of the
chicken β-actin gene called BA-CD18.   Sequences 5' to the
immediate early (IE) gene of human cytomegalovirus were
subcloned into PUC19 and a portion containing IE enhancer
sequences was removed on a Xho 1(from the polylinker) to
30   Nco 1(-220 of the IE gene) fragment).   Synthetic linkers
were used to convert the NcoI site to a XhoI site and the
modified fragment was cloned into the unique XhoI site of
BA-CD18 located 5' to the β-actin promoter.   This new

-79-

vector was called CMV-BA-CD18.

Retroviral particle production The SAX retroviral
particles were provided by Dr. Clay Smith in Dr. Eli
Gilboa's laboratory as viral supernatant solutions frozen
5   and stored at -80°C. The DC-29 and CD18 retroviral
particles were produced by growing the DC-29 and CD18 viral
packaging line to near confluency in a T-75 flask, changing
all the medium, incubating the cells for 12-15 hours and
then collecting the medium containing the viral particles.
10   The virus containing supernatant was then centrifuged to
remove vital packaging cells, the medium removed, and
frozen in aliquots at -80°C.

LTBMC with supernatant added SAX retrovirus. DC29
retrovirus, or CD18 retrovirus The cultures were incubated
15   at 37°C in a humidified 5% CO_2/95% air atmosphere. During
the two first weeks in culture, two thirds of the medium (1
ml) was removed from each culture well daily and the medium
replaced with an equivalent volume fresh medium containing
HGF's (0.85 ml) and viral cell producer supernatant; (0.15
20   ml). The retroviral supernatant containing medium was
thawed immediately prior to use and if not completely
urged, it was stored on ice in a refrigerator. The medium
removed from the cultures was centrifuged, the medium
decanted, and the cells returned to the original wells.

25   LTBMC co-cultured with SAX retrovirus packaging cell
line The SAX retrovirus packaging cell line was grown to
approximately 10% confluency in T-25 flasks (Costar, No.
3056) and then subjected to 2000 rads of radiation. The
hematopoietic cells prepared above were added to the
30   irradiated viral producer cells and cultured wit 50% daily
medium exchange for 2.5 weeks with all cells being returned
to the wells. At 2.5 weeks in culture, a 0.5 mM solution

-80-

of EDTA was added to the flasks to remove the hematopoietic
cells while leaving the stroma. The removed hematopoietic
cells were added to 3 wells of a 6 well plate with 1000
freshly trypsinized bone marrow fibroblast cells per well.

5   Sampling of infected LTBMCs Beginning at week two in
culture, after retrovirus addition had ended or co-culture
had ceased, the cultures had 50% medium exchanged per day
with the nonadherent cells in the exchanged medium being
removed once per week for analysis. Nonadherent cells were
10   removed from the cultures during the daily medium exchange,
the mononucleated cells counted, and fresh medium returned
to the wells. The remaining six days per week when the
cells were not counted, 50% of the medium was removed from
each of the culture well sand replaced with fresh medium,
15   the removed was centrifuged, the medium decanted from the
cell pellet, and the cells returned to their original
wells.

       Analysis for retroviral infection The initial bone
marrow inoculum was plated in 0, 0.4, 0.8, 1.2, 1.6, and
20   2.0 mg/ml G418 to obtain a kill curve to determine the
concentration of G418 in which to plate the post-infected
bone cells. Cells removed from the cultures were plated in
methylcellulose with G418 at 0.0, 0.8, and 1.6 mg/ml G418.
After two weeks, the number of progenitor cell colonies
25   were enumerated in the methylcellulose. Individual
colonies were then plucked from the methylcellulose and
assayed by polymerase chain reaction (PCR) for retroviral
DNA.

       Statistical analysis The probability of significant
30   difference between groups of cultures was determined by
comparing the cumulative cell production values from the
experimental samples to the matched control cultures using

-81-

a paired t-test. Statistical significant was taken at the
5% level.

Results:
Retroviral infection using SAX retrovirus
 5   Kinetics of cell production in LTBMCs infected with
SAX retrovirus Cell production in retrovirally-infected
cultures is an indicator of the likelihood of retroviral
infection and is therefore a useful parameter to measure.
Retroviral integration into the target cell genome is only
10   thought to occur during cell division. for this reason,
increased culture productivity increases the probability of
stem cell mitosis and thereby increases the probability of
retroviral infection. The highest cell production occurred
in the cultures with supernatant virus addition
15   supplemented with IL-3+GM-CSF and IL-3+GM-CSF+IL-1α which
produced increasing number of cells through 4 weeks in
culture. The LTBMCs co-cultured with the SAX virus
packaging cells produced more cells than the supernatant
addition cultures at week two, although cell production
20   decreased after week 2.

     Analysis of retroviral infection in LTBMCs with
supernatant SAX virus addition The percentage of
progenitor cells surviving in high [G418] varied from 2 to
50% in the cultures supplemented with IL-3+GM-CSF or IL-
25   3+GM-CSF+IL-1α during the first four to six weeks in
culture. By 10 weeks in culture (8 weeks after virus
addition had ended) 43% of the number of progenitor cells
that were clonable to hematopoietic colonies survived
exposure to G418. This indicates that these progenitor
30   cells had been rendered G418 resistant by virtue of
containing the G418 resistance gene transferred by the
retrovirus to stem cells present in the culture during the
initial 14 day infection period. The rapidly perfused

cultures not supplemented with HGFs had an average 12%
progenitor cell survival in high [G418] between weeks 8 and
11. At culture termination at 11 weeks, the stromal layer
of the IL-3+GM-CSF supplemented cultures was trypsinized

5   and 17% of progenitor cells that were adherent to the
stroma survived in high [G418]. This suggests that a
significant percentage of adherent progenitor cells were
also infected with the SAX virus.

        Analysis of retroviral infection in LTBMCs co-cultured

10   _with irradiated SAX virus packaging cells_ The percentage
of progenitor cells surviving in G418 when co-cultured with
irradiated SAX cells varied between 0 and 36%. The
cultures not supplemented with HGFs produced CFU-GM that
survived in high [G418] only between weeks 4-7. After week

15   7, no CFU-GM were produced in these cultures that survived
in high [G418], suggesting that little or no infection of
stem cells occurred. The LTBMCs supplemented with IL-3+GM-
CSF and co-cultured with irradiated SAX cells for 2.5 weeks
produced high percentages of CFU-GM that survived in 0.8

20   mg/ml G418 at weeks 4, 5 and 8. However, at week 10, these
cultures failed to produce CFU-GM that were resistant to
G418. This suggests that little or no infection of stem
cells occurred in these cultures or that the stem cells may
have differentiated or died.

25   _Retroviral infection using DC-29 retrovirus_
        Kinetics of cell production in LTBMCs infected with
DC-29 retrovirus The number of cells produced in the
cultures infected with DC-29 retroviral supernatant was HGF
dependent. The cultures supplemented with IL-3+GM-CSF+Epo

30   produced between 1.5-4 x 10^6 cells on a weekly basis
throughout the 10 weeks of culture. The cultures
supplemented with IL-3+GM-CSF+Epo+MGF were more prolific,
while the cultures supplemented with Hybrikine+Epo resulted

in the highest cell production. Interestingly, the control
cultures supplemented with IL-3+GM-CSF+Epo but not
receiving DC-29 retroviral supernatant additions were less
prolific than the similar cultures receiving the DC-29
5   retroviral supernatant. Cell production in the IL-3+GM-
CSF+Epo, IL-3+GM-CSF+Epo+MGF and Hybrikine+Epo cultures
(with virus addition) was significantly higher than the
control culture (IL-3+GM-CSF+Epo, no virus addition) at the
5%, 1% and 1% level of significance, respectively. A part
10   of the increased production in the cultures with retroviral
supernatant addition may be due to the presence of a growth
factor(s) such as MGF (c-kit ligand) which is known to be
produced by the 3T3-based packaging cell line.

     Analysis of retroviral infection in LTBMCs with
15   supernatant DC-29 virus addition The efficiency of
retroviral infection was assessed by CFU-GM survival in 1.6
mg/ml G418, a concentration that killed all bone marrow
cells prior to retroviral infection. The average percent
of CFU-GM surviving at week 8 (6 weeks after infection) was
20   high in all infected cultures, Table 5.

Table 5: Percent of CFU-GM surviving in 1.6 mg/ml G418 after infection with DC-29 retrovirus as a function of HGF supplementation. The cultures were infected with DC-29 retroviral containing supernatent for the initial 2 weeks in culture.

week	IL-3+GM-CSF+Epo with virus					IL-3+GM-CSF+Epo+HGF with virus					Hybrikine+Epo with virus				
	1	2	3	±dev.	ave.	1	2	3	±dev.	ave.	1	2	3	±dev.	ave.
2	4.70	2.40	5.60	1.6	4.2	3.70	21.6	19.5	9.7	1.49	9.00	6.20	18.4	6.4	11.2
4	2.5	0.0	4.6	2.5	2.4	8.1	6.0	16.1	3.3	10.1	9.0	6.2	18.4	5.4	4.4
6	0.0	0.0	0.0	0.0	0.0	1.4	1.7	4.0	1.4	2.4	0.0	0.0	0.0	0.0	0.0
8	0.0	22.2	*	12.8	7.4	0.0	14.5	11.5	3.6	9.0	21.6	14.7	10.7	5.5	15.7
10	*	0.0	*	0.0	0.0	8.2	0.0	0.0	4.7	2.7	0.0	0.0	0.0	0.0	0.0
10†	2.6	0.0	*	1.5	0.9	7.7	0.0	*	4.4	2.6	0.0	0.0	0.0	0.0	0.0

* N/A because no CFU-GM were counted in 0.0 mg/ml G418.

† % CFU-GM surviving in 1.6 mg/ml G418 from the trypsinized stroma in each culture at experiment termination

% survival was calculated as the (number of CFU-GM at 1.6 mg/ml G418 - ave. number of CFU-GM at 1.6 mg/ml G418 in uninfected cultures) divided by (number of CFU-GM at 0.0 mg/ml G418) * 100

Addition of MGF to the combination of IL-3+GM-CSF+Epo
appears to have increased infection efficiency at weeks 8-
10 in culture, with one of the cultures containing 7.7%
G418 resistant colonies. The data at week 8 suggests that
5   the cultures supplemented with Hybrikine+Epo had a high
infection efficiency, although the data at week 10 suggests
no infection. At week 10, note that the number of CFU-GM
removed from several cultures decreased to where few or no
CFU-GM would be expected be seen at approximately 10%
10   infection. In addition, 1.5 mg/ml G418 is an extremely
high antibiotic dose, enough to likely overwhelm the
efficacy of the G418 resistance gene transfected at even
slightly suboptimal levels. Thus, the efficiency of gene
transfer into the hematopoietic stem cells in these
15   cultures was at least 7.7% in some samples, and perhaps as
high as 45% or higher.

Kinetics of nonadherent progenitor cell production
Because analysis of retroviral infection in these
experiments depends on assaying for progenitor cells to
20   infer stem cell existence, infection, and cycling, it is
important to determine the effect of HGFs on progenitor
cell production in culture. Also, in this retroviral
experiment, MGF and Hybrikine are being used in combination
with other HGFs. Both MGF and Hybrikine have not been used
25   in rapidly perfused HGF supplemented LTBMCs, and it is
therefore necessary to determine their effects on
hematopoiesis.
Progenitor cell production was strongly dependent on
HGF supplementation. The number of CFU-GM removed from the
30   cultures is shown in Table 6.

Table 6:   The cumulative number of progenitor cells
                  removed in DC-29, HGF supplemented rapidly
                  perfused human long-term bone marrow
                  cultures.

Culture	Virus Added	Total Number of CFU-GM Removed
Innoculum	N/A	900
Control	No	1300
(IL-3+GM-CSF+Epo)		
IL-3+GM-CSF+Epo	Yes	2800
IL-3+GM-CSF+Epo+MGF	Yes	5400
Hybrikine+Epo	Yes	5000

Every second weekly sampling from the cultures was assayed
for CFU-GM and the non assayed values were estimated by
linear interpolation between two known data points. The
known and interpolated values were summed to approximate
the total number of CFU-GM removed from culture.

        Addition of retroviral supernatant increased
progenitor cell removal 2.2-fold over no addition of
retroviral supernatant. The increase in the number of CFU-
GM removed in the cultures supplemented with retroviral
supernatant and IL-3+GM-CSF+Epo+MGF or Hybrikine+Epo was
4.2 and 3.8-fold greater then the uninfected control,
respectively. Removal of CFU-GM was statistically greater,
at the 1% level of significance, in all viral supplemented
cultures when compared to the number of CFU-GM inoculated.

Progenitor cells production in culture

        A population balance on the CFU-GM compartment shows
that the addition of MGF or Hybrikine to cultures

-87-

supplemented with IL-3+GM-CSF+Epo have a significant
positive effect on the CFU-GM pool. The addition of MGF to
the combination of IL-3+GM-CSF+Epo increased CFU-GM removal
1.9-fold and CFU-GM differentiation 0.5-fold compared to
5   similar cultures not supplemented with MGF, Table 7.

Table 7:   The cumulative production of CFU-GM removed and
            differentiated in growth factor supplemented DC-
            29 retroviral infected rapidly perfused human
            long-term bone marrow cultures.

Culture	Number of CFU-GM removed (plated)	Number of GM cells removed ($x10^{-6}$)	Estimated number of CFU-GM that differentiated	Total number of CFU-GM
Innoculum control (IL-3+GM-CSF+Epo, no virus added)	(900) 1300	(0) 1.1	(0) 1100	(900) 2400
IL-3+GM-CSF+Epo (virus added)	2800	1.8	1800	4600
IL-3+GM-CSF+Epo+MGF (virus added)	5400	3.1	3100	8400
Hybrikine+Epo (virus added)	5000	6.1	6100	11,100

Every second weekly sampling from the cultures was assayed for CFU-GM and
the non assayed values were estimated by linear interpolation between two
known data points. The known and interpolated values were summed to
approximate the total number of CFU-GM removed from culture.

The combination of Hybrikine+Epo had a very pronounced
effect on CFU-GM production and differentiation.
Hybrikine+Epo induced a 1.8-fold increase in CFU-GM removal
and over a 3-fold increase in CFU-GM differentiation
compared to the previous optimum cultures supplemented with
IL-3+GM-CSF+Epo. Also, Hybrikine+Epo induced production of
almost twice the number of granulocytes and macrophages
than did the combination of IL-3+GM-CSF+Epo+MGF. This
indicates that Hybrikine is a potent inducer of the

-88-

granulocyte macrophage lineages.

<u>Analysis of neutrophils produced from stem cells</u>
<u>infected with CD18 encoding retrovirus</u> CD18 deficient bone
marrow was enriched for early hematopoietic cells as
5 described above, and then cultured for 14 days with 50%
daily medium exchange supplemented with 1.0 ng/ml/day GM-
CSF and 1.0 ng/ml/day IL-3 and 40 U/ml/day IL1α, and with
CD18 retroviral producer line supernatant. From day 15 on,
the cells were cultured under the same conditions without
10 addition of retroviral supernatant. Nonadherent cells were
removed from the cultures weekly and analyzed for the
presence of cell surface CD18 by flow cytometry using a
biotinylated anti-CD18 monoclonal antibody by standard
methods (Updyke et al Meth. Enzymol. (1986) 121:717-725).
15 Whereas CD18 deficient bone marrow cells failed to express
any cell surface CD18 protein by this assay, neutrophils
and monocytes that arose from the retrovirally infected
cultures did express cell surface CD18. In triplicate
cultures, expression of cell surface CD18 was 3.5%/5%/2% at
20 6 weeks and 11%/28%/3% at 11 weeks. Since the neutrophils
and monocytes present in the cultures at 11 weeks arose
form CFU-GM progenitor cells only 10-14 days earlier, these
data indicate that human hematopoietic stem cells were
successfully and stably transfected with the recombinant
25 retrovirus during the first two weeks of the culture.

In interpreting the present results it is important to
recognize that although several groups have demonstrated
retroviral-mediated gene transfer into human hematopoietic
progenitor cells, gene transfer into human hematopoietic
30 stem cells has not been shown. The rapid decay of cell
production in traditional, slowly perfused human LTBMCs
limited infection determination due to the absence of
progenitor cells required for the assay.

-89-

Retroviral infection in the present studies was initially assessed by growing cells removed from the LTBMCs in methylcellulose in the presence of the mammalian cell antibiotic G418. Progenitor cells infected with the SAX or
5   DC-29 retroviruses and expressing the NEO product will be able to survive and form colonies in high concentrations of G418, whereas noninfected cells will die in high concentrations of G418. Also, production of progenitor cells that survive in high concentrations of G418, six or
10   more weeks after virus addition has ended, requires those cells to have recently differentiated from a more primitive cell (a stem cell) and therefore suggests that stem cells were infected. Similarly, the CD18 expressed on the surface of neutrophils and monocytes produced during the
15   11th week of culture requires that a primitive hematopoietic stem cell was infected during the first 2 weeks of the cultures, because all mature cells, precursors, and clonogenic progenitors present during the infection period had died by 4-5 weeks in the culture.

20   The analysis for retroviral infection used here can underestimate the percentage of cells infected with the retrovirus due to insufficient expression of the NEO gene product. Under-expression of the transferred gene product has been shown to be a problem in human and primate models.
25   Therefore, the percentage of progenitor cells infected in this study is probably a conservative estimate.

The percentage of progenitor cells that survived in high [G418] was approximately 40% at week 10 in the cultures infected SAX retrovirus supernatant and
30   supplemented with IL-3+GM-CSF±IL-1α. These initial results show that a high percentage of stem cells were infected in the cultures supplemented with retroviral supernatant during the first two weeks in culture.

-90-

The percentage of progenitor cells infected with the
DC-29 retrovirus was high (0-21%) in the IL-3+GM-
CSF+Epo+MGF and Hybrikine+Epo cultures during the initial 4
weeks after viral addition had ended. This high level of
5   progenitor cell infection was probably due to direct
infection of progenitor and primitive cells by the
retrovirus. The percentage of progenitor cells surviving
in high G418 concentration decreased 4 weeks after viral
addition had ended, but rebounded 2 weeks later to 0-22%
10   survival in high G418 concentration.

The production of G418 resistant progenitor cells in
the DC-29 experiment may underestimate the percentage of
progenitor cell actually infected with the DC-29
retrovirus. The high concentration of G418 used to select
15   for infected colonies (1.6 mg/ml of G418) is twice as high
as that used for the SAX infection experiment and requires
high expression levels of the NEO gene product, neomycin
phosphotransferase, to survive.

Interestingly, in the LTBMCs supplemented with either
20   the SAX or DC-29 retrovirus containing supernatant, the
HGFs IL-3+GM-CSF±Epo, IL-1α, or MGF or the combination of
Hybrikine+Epo, the percent of progenitor cells surviving in
G418 increased 6-8 weeks after viral addition ended.
Although the mechanism for the increase in the percentage
25   of G418 surviving progenitor cells towards the latter
stages of the culture are not known one possibility is that
stem cells that were infected during the first two weeks in
culture became more active as the culture progressed. This
would effectively increase the percentage of cells
30   surviving in G418. Another possibility is that expression
of the NEO gene may have increased in progenitor cells
produced late in culture due to differentiation from a stem
cell which had a different and better expressible

integration site than did progenitor cells transfected
directly during the initial infection culture period.
Therefore, the high level of progenitor cell survival late
in culture although possibly due to several causes, does
5 strongly suggest that stem cells were infected in these
LTBMCs.

   In sum, these data document that under the culture
conditions disclosed in the present invention,
hematopoietic stem cells were proliferating in the
10 cultures, permitting the incorporation of retrovirally
transferred genetic material into these cells. Progenitors
were continuously and actively produced from these stem
cells, and these many of these progenitors contained and
expressed the transfected genes. These data indicate that
15 genetically modified human hematopoietic stem cells were
present and proliferating in these cultures.

IV. **EXPERIMENTAL**

I. **Formation of Transformants**

   The growth factor human GM-CSF (Wong, Science (1984)
20 228:810-815) was inserted into a eukaryotic expression
vector. The hGM-CSF cDNA (EcoRI to AhaIII, approximately
700 bp fragment) was cloned into an EcoRI to PstI fragment
of pSP65. (Melton, Nucl. Acids Res. (1984) 2:7035-7056).
The resulting plasmid was SP65GM-CSF. The mouse
25 metallothionein promoter (Glanville, Nature, (1981)
292:267-269) was digested with EcoRI and BglII and the
approximately 2 kb fragment containing the promoter was
inserted into the EcoRI to BamHI fragment of pSP65 to make
p65MT. The plasmid pMT GM-CSF was then constructed by
30 digesting pSP65GM-CSF with EcoRI, filling in the overhang
with the Klenow fragment of DNA polymerase I and then
digesting the resulting linearized DNA with HindIII to
isolate the 700 bp fragment comprising the coding region of

-92-

GM-CSF. This fragment was subcloned into the SalI
filled/HindIII site of p65MT. The 2.7 kb fragment
comprising themetallothionein promoter and the GM-CSF
coding region was then isolated and placed into pSV2neo

5   (Southern and Berg, J. Mol. Appl. Genet (1982) 1:327) from
which the SV-40 promoter was removed. This results in the
SV-40 poly A signal downstream of the GM-CSF coding
sequence.

     The neomycin resistant gene, which confers resistance

10   to the antibiotic gentamicin (G418) was taken from pSV2neo
by isolating the approximately 3 kb PvuII to EcoRI fragment
and placing EcoRI linkers onto the PvuII site. The neo
resistance gene with EcoRI ends was subcloned into the
EcoRI site of the GM-CSF expression plasmid to create the

15   plasmid MTGM-CSFneo.

     The plasmid MTGM-CSFneo alone and as a cotransfection
with the plasmid (Yang, Cell (1986) 47:3-10) encoding the
gibbon ape IL-3 gene under the control of the SV-40
promoter and poly A site, were transfected by

20   electroporation of linearized DNA into the African green
monkey cell line CV1 and the mouse cell line NIH 3T3 cells.
Transformants were selected by selection in media
containing 500 mg/ml of G418, isolated, and screened for
production of GM-CSF or IL-3 by bioassay of supernatants

25   using AML-193 cells (Adams et al, Leukemia (1989) 3:314).
Several of the positive lines were then employed as stroma
for human bone marrow cells in Dexter culture.

     In addition, normal mouse bone marrow cellswere
transfected with the above plasmids using the

30   calcium/phosphate method of Okayama (Chen, Mol. Cell. Biol.
(1987) 7:2745-2752) and were found to efficiently express
the introduced genes.

GM-CSF and IL-3 secretion by the transfected
fibroblasts was investigated. Serum free 72 hour culture
supernatants were obtained from the NIH-3T3 cells and
assayed for hGF secretion by ^{3}H uptake on target cells

5   inhibitable by neutralizing rabbit anti-GM-CSF or anti-IL-3
antibodies. Proliferation induced by 20 mg/ml GM-CSF was
set as 100 units GM-CSF and that induced by 10 ng/ml IL-3
was set as 100 units IL-3. The co-transfected cells
produced about 35 units/ml of GM-CSF and about 57 units/ml

10   of IL-3.

II. <u>Perfusion Chamber</u>

The perfusion chamber is a glass cylinder with Delrin
caps to allow for autoclaving without deformation and
biocompatability. The caps have cylindrical groves into

15   which the glass cylinder fits. At the bottom of the grove
an O-ring is placed to seal the lumen of the chamber. The
caps have several holes into which Luer (Luer Lok) fittings
are provided into which media andgas delivery lines are put
as well as an extended tube into the central section of the

20   chamber to sample adherent and/or non-adherent cells. The
caps are attached with three long bolts, spaced 120°,
placed outside the glass cylinder; wing nuts and washers
are used to tighten the assembly.

The chamber is hooked to a side reservoir. The loop

25   contains a pump, a chamber of on-line sensors, oxygenator,
and sample and injection ports in addition to the side
media reservoir. The media in the side reservoir is then
slowly exchanged using a separate pump. This configuration
allows for separate control of the media exchange rate and

30   the flow rate through the oxygenator and perfusion chamber.
The former is used to control the longer term change in the
media composition and perfusion, while the latter may be
used to control the dissolved oxygen tension and flow

patterns in the chamber. The use of a small mesh
polysulfonate membrane allows for plug flow in the chamber
and the precise control of delivery of growth factors and
other special compounds which one may wish to introduce

5   into the bioreactor in very precise amounts.

The transfected stromal cells are seeded either over a
bed of shredded collagen sponge or the stromal cells are
placed on one side of a 5μ porous polycarbonate filter
precoated with collagen and the stromal cells allowed to

10   adhere to the filter over a number of hours. The cells are
allowed to grow in an appropriate nutrient medium until the
cells become confluent on one side while sending
cytoplasmic projections through the pores. Bone marrow
cells are then seeded on the other side of the membrane and

15   the stem cells attach to the intruded cytoplasmic
projections which have passed through the pores.

After autoclaving the chamber and components of the
loop, the reactor is assembled in a sterile environment.
The media is then circulated through the side loop and

20   chamber for a few days while signs of contamination are
monitored. The central section of the bioreactor is then
inoculated with either the extra-cellular matrix alone or a
preinoculated extracellular matrix support that contains
the stromal cells. The stromal cells may then be kept in

25   the chamber for a period of a few days while their
metabolic performance and/or growth factor responsiveness
is monitored and if results are satisfactory, the bone
marrow is inoculated or immediately seeded with bone
marrow. In either case, the cell layer is kept at the

30   bottom of the central section of the perfusion chamber.

The cells lay down additional extra-cellular matrix
and the cell layer adheres to the support. Where the

-95-

membrane is used, the chamber may be inverted and the cell
layer is then located at the ceiling of the central
section. In this configuration, the maturing cells settle
on the bottom of the central chamber asthey loose their
5   adherence to the stromal layer. The non-adherent cells are
then harvested by constant cell flow, driven by the medium
perfusion pressure, into the exit tubing.

In a typical run, the chamber was inoculated with
NIH-3T3 cells on day one on shredded collagen sponge
10   support. For the first 40 days perfusion rates and other
operating variables were adjusted. At day 40 a reasonable
steady state was achieved which was maintained for about 20
days. On day 64 the chamber was seeded with 33×10^6 human
bone marrow cells. For the first 10 days the harvested
15   cell count decreased until it settled in a steady state of
about $7-8 \times 10^5$ cells produced every three days. Flow
cytometric analysis showed that a constant fraction, about
20% of the harvested cells were HLA-DR positive. On day 90
a pump failure was experienced and the pH dropped below 6.9
20   overnight. When the perfusion rate was restored the
production of non-adherent cells recovered and was
approaching the previous steady state production rate when
a bacterial contamination occurred. At this point, the
study was terminated.

25   The above results demonstrated that a perfusion
chamber is capable of performing ex vivo hematopoiesis,
hematopoiesis may be restored ex vivo after a pH drop, the
glucose concentration data showed that the hematopoietic
cells grow primarily aerobically on glucose, since the
30   glucose concentration drops after inoculation without
increasing the lactate concentration indicating that
oxygenation is limiting. The glucose/lactate (anaerobic)
metabolism appears to be primarily due to the NIH-3T3

-96-

stromal bed. Similarly, the glutamine and ammonia
concentrations reach preinoculum levels once the
hematopoietic cell number levels off, implying that the
glutamine consumption by the bone marrow cells is much less
5   than that of the stromal bed.

III.   Monitoring of Metabolic Products

The consumption and formation rates of glucose and
lactate as well as glutamine and ammonia were determined
for transfected NIH-3T3 cells.   (The medium was IMDM plus
10   20% FCS).   Increased glucose consumption was only observed
for daily fed T-flasks, whereas all less frequently fed
cultures follow the same slowly diminishing glucose uptake
rate pattern.   Cultures that were exchanged 50% daily were
switched to the 100% daily exchange schedule on day 18,
15   which resulted in an immediate increase in glucose
consumption following the same trend as that observed for
cultures exchanged 100% daily from day one.   Lactate
production rates follow a similar pattern, as the lactate
yield on glucose is essentially a constant (0.9
20   lactate/glucose; indicating a largely anaerobic stromal
metabolism).

The glutamine and ammonia concentrations show a
pattern analogous to the glucose/lactate metabolism.   Using
values corrected for chemical decomposition of glutamine at
25   37°C, the glutamine consumption rate versus the glucose
consumption rate shows relative uptake rates are constant,
about 1:8 glutamine: glucose. The predicted optimum ratio
varies with oxygen uptake rate - the ratio drops with
increasing optimum uptake rate.

30   Analogous conclusions were supported by

glucose/lactate metabolic data derived from normal bone
marrow stromal fibroblasts. Under conditions of infrequent
medium exchange the cultures were primarily anaerobic, with
high steady state levels of lactate rapidly achieved and
5   maintained. With more frequent medium exchange, the cell
metabolism became more rapid, with increased glucose
consumption and lactate production. No detectable
consumption of glutamine was observed after correcting the
data for spontaneous chemical decomposition. For both 3T3
10 cells and normal human bone marrow cells, the cells
continue to divide and crowd when the serum/media exchange
rate was above what appears to be a critical replacement
schedule.

To further ascertain the relative importance of
15 perfusion rate of serum versus that of nutrients, the
following experiments were performed: 1) one set of
T-flasks with 20% serum containing media exchanged daily;
2) two sets of T-flasks, one with 20% serum and the media
exchanged every other day and one with 10% serum with the
20 media exchanged daily; 3) two sets of T-flasks, one with
10% serum and the media exchanged every other day, one with
5% serum with the media exchanged daily; 4) two sets of
T-flasks, one with 5% serum and the media exchanged every
other day and one with 2.5% serum with the media exchanged
25 daily. The serum exchange rate is the same within each
group while the exchange rate of the nutrient containing
media varies. The results from these experiments show that
it is the exchange rate of the serum that is critical.
While for the experiment 1) glucose consumption increased
30 and by day four had substantially flattened out to a rate
of about 9.5 mmoles/per day, in all of the other cases, the
glucose consumption started below the original glucose
consumption of Group I and dropped off in a substantially
linear manner regardless of whether twice the amount of

-98-

serum was used and changed every other day or half the
amount of serum was used and the media changed every day.
This supports the need for a critical perfusion rate of
serum or one or more serum components that influence the
5   metabolic growth behavior of the stromal cells.

It is evident from the above results, that one may
grow hematopoietic cells in a bioreactor in an efficient
manner. Stromal cells can be provided from homologous or
heterologous sources, where the stromal cells have been
10   transfected with genes to provide for the important growth
factors. In this manner, serum need not be added to the
media to support the growth of the cells. By providing for
stromal cells which adhere to a support in a manner which
allows for separation of hematopoietic cells from the
15   stromal cells, the hematopoietic cells may be continuously
harvested for use. By appropriate choice of combinations
of factors, specific lineages of hematopoietic cells may be
grown. In addition, if desired, the stromal cells may
provide for a reservoir of transfecting viruses for the
20   introduction of genes into the hematopoietic cells.

All publications and patent applications cited in this
specification are herein incorporated by reference as if
each individual publication or patent application were
specifically and individually indicated to be incorporated
25   by reference.

* * * * *

Obviously, numerous modifications and variations of
the present invention are possible in light of the above
teachings. It is therefore to be understood that within
30   the scope of the appended claims, the invention may be

-99-

practiced otherwise than as specifically described herein.

<u>Claims</u>

1. A method for culturing a cellular composition
containing human stem cell and obtaining *ex vivo* human stem
cell division comprising culturing said cellular
5   composition in a liquid culture medium which is replaced,
either continuously or periodically, at a rate of about 1
ml of medium per ml of culture per about 24 to about 48
hour period, and removing metabolic products and
replenishing depleted nutrients while maintaining said
10   culture under physiologically acceptable conditions.

2. The method of Claim 1, wherein said medium is
replaced continuously.

3. The method of Claim 2, wherein replacement of said
medium comprises perfusing fresh medium through at least
15   part of the mass of said human stem cells.

4. The method of Claim 1, wherein said medium is
replaced periodically.

5. The method of Claim 4, wherein replacement of said
medium comprises perfusing fresh medium through at least
20   part of the mass of said human stem cells.

6. The method of Claim 1, wherein said medium is a
hematopoietic culture medium and at least one member
selected from the group consisting of human peripheral
blood mononuclear cells, human bone marrow cells, human
25   fetal liver cells, and human cord blood cells, are
cultured.

7. The method of Claim 1, comprising:

combining in a reactor vessel stromal cells adherent

to a packing and human hematopoietic cells comprising stem
cells, wherein at least a portion of said stromal cells are
transformed and capable of excreting at least one growth
factor which directs the proliferation, differentiation, or
5   both, of said hematopoietic cells;

       substantially continuously perfusing said cells in
said reactor with a nutrient medium while removing
metabolic products and replenishing depleted nutrients, and
while maintaining said reactor under physiologically
10   acceptable conditions; and

       harvesting hematopoietic cells from said reactor, with
the proviso that when said human hematopoietic cells
inoculated into said reactor vessel are suspected of
comprising neoplastic cells, said perfusing is at a rate
15   providing a force greater than the affinity of neoplastic
cells to said stromal cells and less than the affinity of
normal hematopoietic cells.

       8.   The method of Claim 7, comprising using a
perfusion rate resulting in a shear stress at the surface
20   of said hematopoietic cells greater than about 1.0
dyne/cm^2.

       9.   The method of Claim 1, comprising culturing said
cellular composition in the presence of a gene transfer
vector and obtaining a stable, genetically transformed
25   human stem cell.

       10.   The method of Claim 9, wherein said medium is
replaced continuously.

       11.   The method of Claim 10, wherein replacement of
said medium comprises perfusing fresh medium through at

least part of the mass of said human stem cells.

12.   The method of Claim 9, wherein said medium is
replaced periodically.

13.   The method of Claim 12, wherein replacement of
5   said medium comprises perfusing fresh medium through at
least part of the mass of said human stem cells.

14.   The method of Claim 9, wherein said medium is a
hematopoietic culture medium and at least one member
selected from the group consisting of human peripheral
10   blood mononuclear cells, human bone marrow cells, human
fetal liver cells, embryonic stem cells and human cord
blood cells, is cultured.

15.   The method of Claim 9, wherein said cellular
composition has been enriched for said stem cells.

15   16.   The method of Claim 9, wherein said cultured
human stem cells produce stable, genetically transformed
human progenitor cells.

17.   The method of Claim 9, wherein said cultured
human stem cells produce stable, genetically transformed
20   human hematopoietic progenitor cells.

18.   The method of Claim 9, wherein IL-3 and GM-CSF
are added continuously to said medium, each at a rate of
0.1 to 100 ng ml^{-1} day^{-1}.

19.   The method of Claim 18, mast cell growth factor
25   is added to said medium at a rate of from 1 to 100 ng ml^{-1}
day^{-1}, or IL-1α is added to said medium at a rate of from 10
to 100 U ml^{-1} per 3 to 5 day period.

20.   The method of Claim 19, wherein mast cell growth factor is added to said medium.

21.   The method of Claim 19, wherein IL-1α is added to said medium.

5   22.   The method of Claim 19, wherein both mast cell growth factor and IL-α are added to said medium.

23.   The method of Claim 9, wherein said medium comprises animal sera or plasma.

24.   The method of Claim 9, wherein said media
10   comprises a corticosteroid.

25.   The method of Claim 9, comprising maintaining glucose concentration in said medium in the range of from 5 to 20 mM, lactate concentration in said medium below about 35 mM, glutamine concentration in said medium in the range of from 1 to 3 mM, and ammonia concentration in said medium
15   below 2.5 mM.

26.   The method of Claim 14, said culture comprising human stromal cells in an amount of 10^{-3} to 10^{-1} (stromal cells/total cells).

20   27.   The method of Claim 9, wherein said gene transfer vector is a retrovirus.

28.   The method of claim 9, comprising culturing said human stem cells in the presence of a retroviral packaging cell line supernatant.

25   29.   The method of Claim 9, comprising culturing said human stem cells in the presence of a retroviral packaging

-104-

cell line.

30.   A stable genetically transformed human stem cell.

31.   The stable genetically transformed human stem cell of Claim 30, wherein said cell is a human stem cell found in human bone marrow.

32.   The stable genetically transformed human stem cell of Claim 30, wherein said cell is a human hematopoietic stem cell.

33.   The method of Claim 1, wherein IL-3 and GM-CSF are added continuously to said medium, each at a rate of 0.1 to 100 ng ml^{-1} day^{-1}.

34.   The method of Claim 33, wherein Epo is added to said medium at a rate of 0.001 to 10 U ml^{-1} day^{-1}, mast cell growth factor is added to said medium at a rate of from 1 to 100 ng U ml^{-1} day^{-1}, or IL-1 (α or β) is added to said medium at a rate of from 10 to 100 U ml^{-1} per 3 to 5 day period.

35.   The method of Claim 33, wherein Epo is added to said medium at a rate of 0.001 to 10 U ml^{-1} day^{-1}.

36.   The method of Claim 34, wherein at least one member selected from the group consisting of G-CSF, basic fibroblast growth factor, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, PDGF and EGF, is added to said medium.

37.   The method of Claim 1, wherein said medium comprises animal sera or plasma.

38.   The method of Claim 1, wherein said media

comprises a corticosteroid.

39. The method of Claim 1, comprising maintaining glucose concentration in said medium in the range of from 5 to 20 mM, lactate concentration in said medium below about 35 mM, glutamine concentration in said medium in the range of from 1 to 3 mM, and ammonia concentration in said medium below 2.5 mM.

40. The method of Claim 6, said culture comprising human bone marrow stromal cells in an amount of 10^{-3} to 10^{-1} (stromal cells/total cells).

41. The method of Claim 1, comprising obtaining an expanded human stem cell pool.

42. The method of Claim 41, comprising obtaining an expanded human hematopietic stem cell pool.

43. The method of Claim 1, comprising culturing human hematopoietic progenitor cells.

44. The method of Claim 43, wherein said cellular composition has been enriched for said human hematopoietic progenitor cells.

45. The method of Claim 43, wherein IL-3 and GM-CSF are added continuously to said medium, each at a rate of 0.1 to 100 ng ml^{-1} day^{-1}.

46. The method of Claim 45, wherein Epo is added to said medium at a rate of 0.001 to 10 U ml^{-1} day^{-1}, mast cell growth factor is added to said medium at a rate of from 1 to 100 ng ml^{-1} day^{-1}, or IL-1 (α or β) is added to said medium at a rate of from 10 to 100 U ml^{-1} per 3 to 5 day

-106-

period.

47. The method of Claim 6, whereby a cell culture
containing red blood cells is obtained.

48. The method of Claim 6, whereby a cell culture
5   containing granulocytes is obtained.

49. The method of Claim 40, whereby the metabolism or
GM-CSF secretion or IL-6 secretion of said human stromal
cells is increased.

50. The method of Claim 49, wherein said human
10   stromal cells are human bone marrow stromal cells.

51. A bioreactor comprising:

a reactor chamber;

means for introducing and removing a nutrient medium
from said reactor chamber and means for monitoring the
15   effluent from said reactor chamber;

in said reactor chamber, stromal cells adherent to a
packing with human hematopoietic cells comprising stem
cells, wherein at least a portion of said stromal cells are
transformed and capable of excreting at least one growth
20   factor which directs the proliferation, differentiation, or
both, of said stem cells.

52. Transformed fibroblast cells comprising a DNA
expression construct capable of expressing at least one
human growth factor in a form capable of excretion, which
25   growth factor directs the proliferation, differentiation,
or both, of progenitor hematopoietic cells.

53. A method of separating hematopoietic neoplastic
cells from normal cells comprising:

combining a cell population of hematopoietic cells
with stromal cells, wherein said stromal cells have limited
5   mobility and said hematopoietic cells contact said stromal
cells; and

Subjecting said hematopoietic to a fluid flow
producing a force at least sufficient to remove neoplastic
cells from contact with said stromal cells, without
10   significant removal of normal cells.

54. The method of Claim 1, comprising removing mature
cells from said culture.

55. The method of Claim 42, comprising removing mature
cells from said culture.
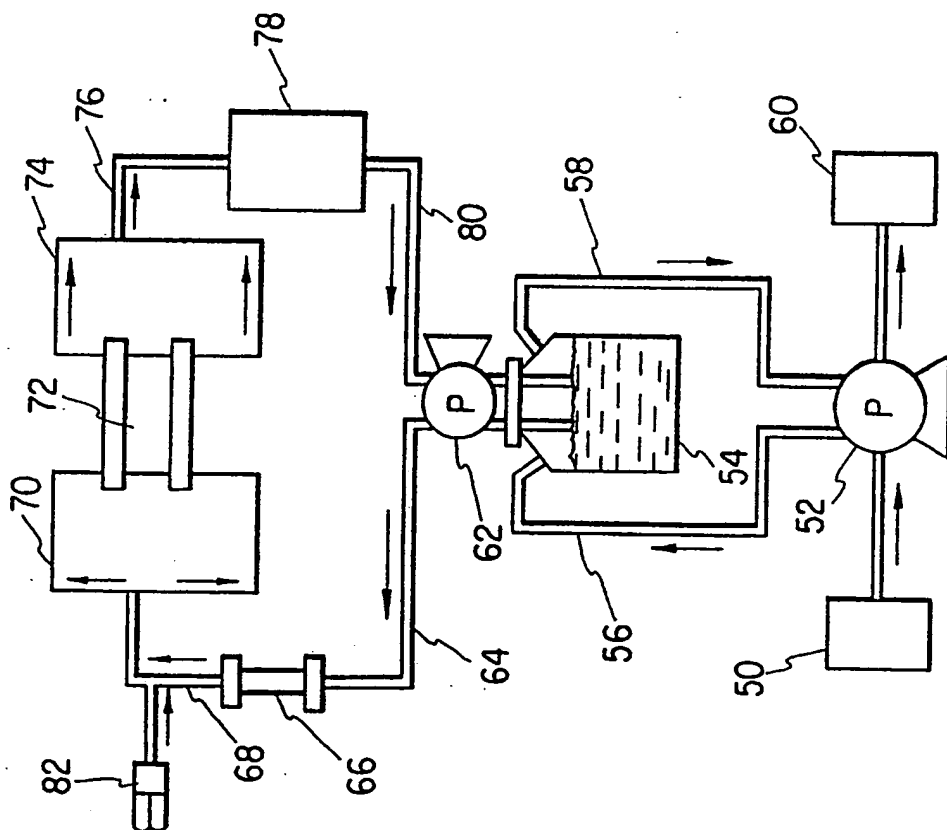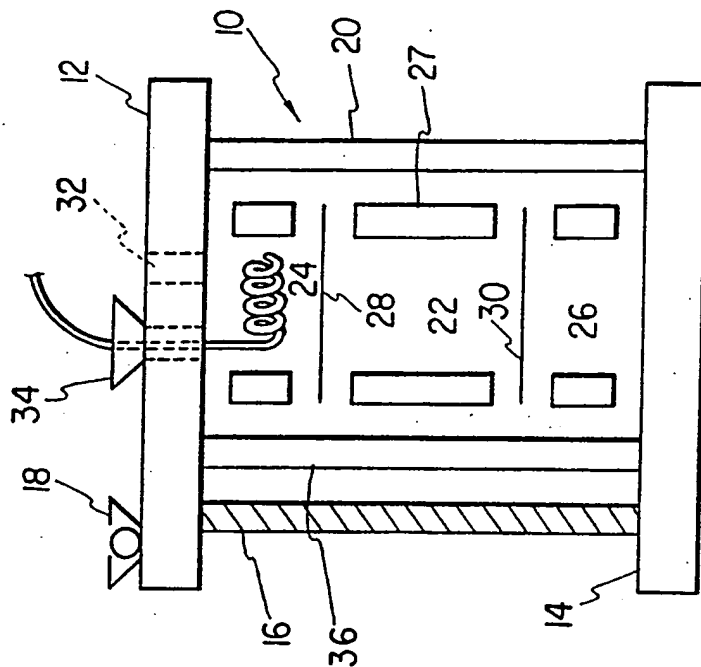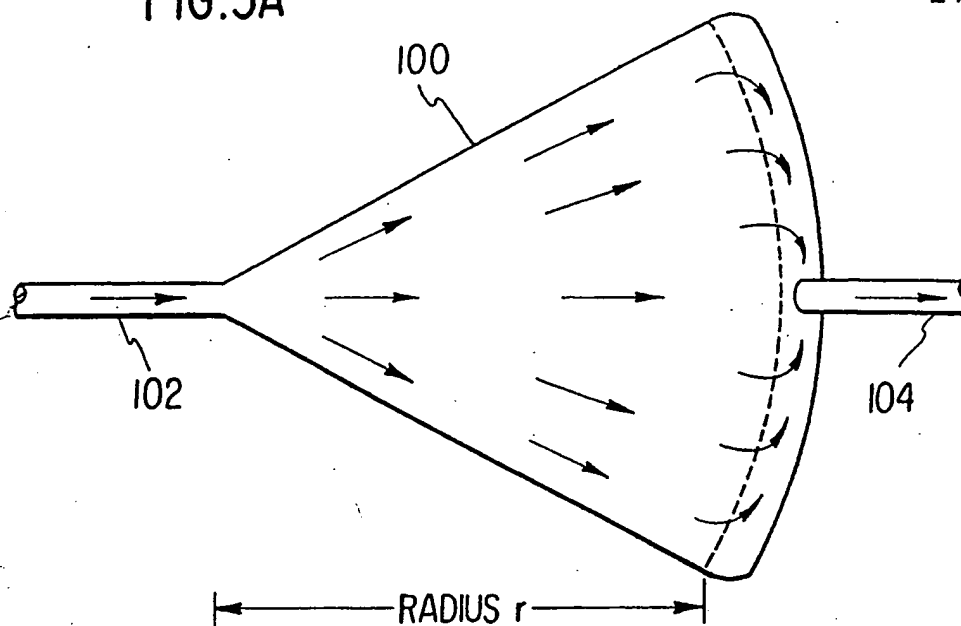
1/4

FIG.1

FIG.2

FIG.3A

2/4

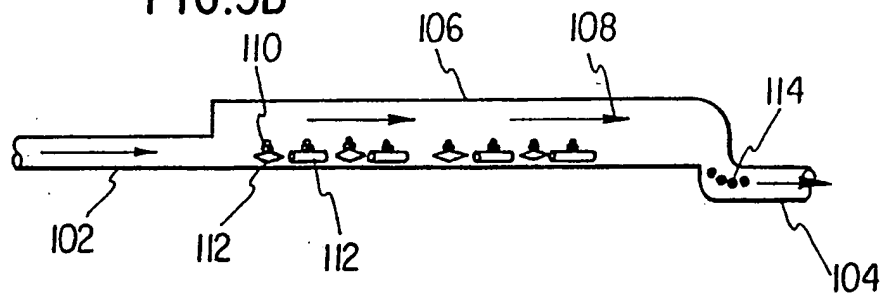100

102

104

RADIUS r

FIG.3B

110   106   108   114

102   112   112   104

FIG.3C

SHEAR STRESS PROFILE

SHEAR
STRESS

P_2

P_1

RADIUS (r)

SUBSTITUTE SHEET
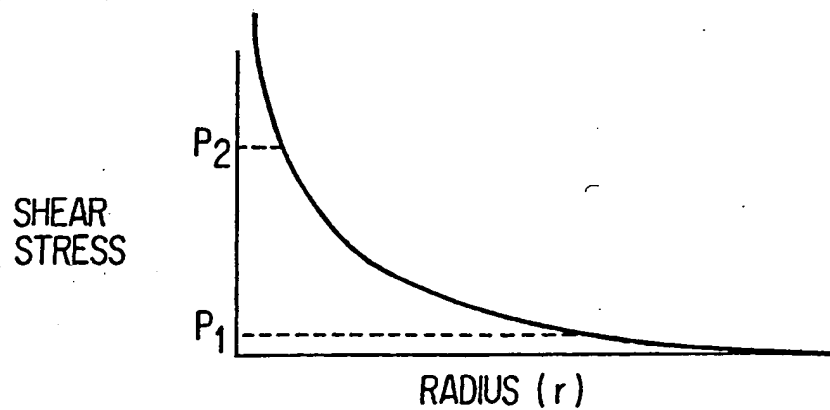
FIG.4A
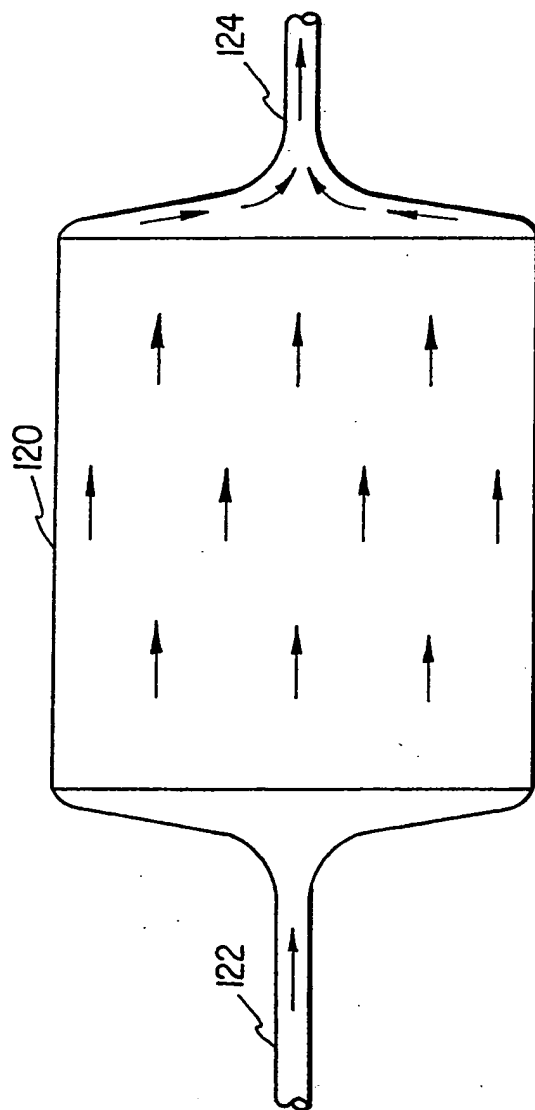
FIG.4B
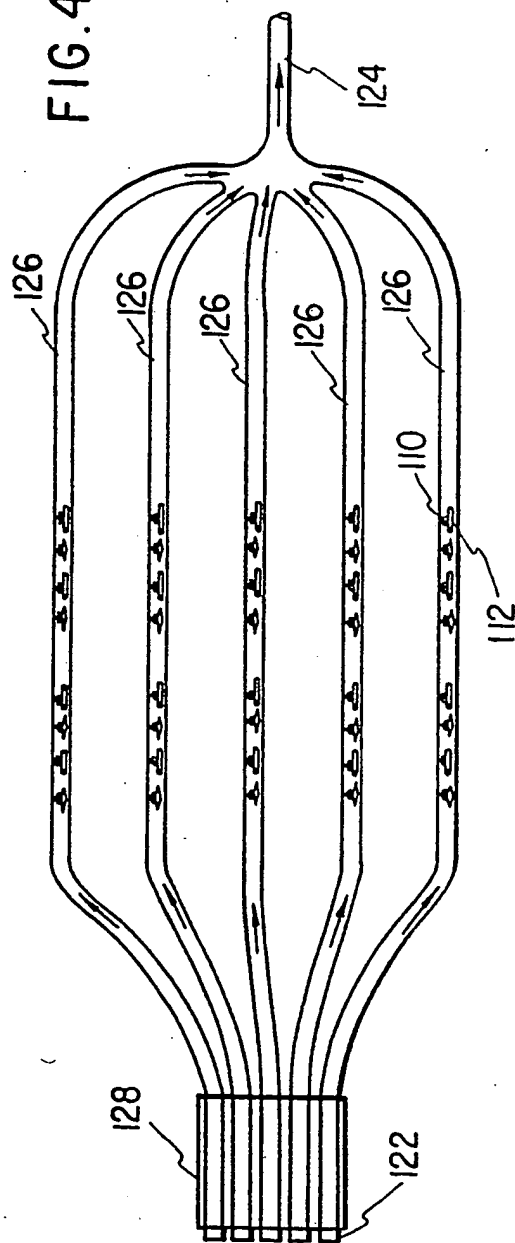
FIG.5A

FIG.5B

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): C12N 5/02, 5/00, 15/00; C12M 3/00, 2/02, 3/04
US CL : 435/240.25, 240.23, 240.241, 284, 285, 286, 172.3

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	435/240.25, 240.23, 240.241, 284, 285, 286, 172.3

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

APS, DIALOG

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category[*]	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
Y	Immunobiology, volume 166, issued 1984, Bodeker et al, "Production of five human lymphokines (Granulocyte-Macrophage Colony Stimulating Factor, Interferon γ, Interleukin-2, Macrophage Cytotoxicity Factor and Macrophage Migration Inhibiting Factor) from ConA stimulated lymphocyte. cultures in bioreactors", pages 12-23, see entire article, especially the abstract.	1 - 6 , 1 0 - 15,18,23,24,25 ,26,33,35- 45,47,48,50,51 ,54,55
Y	US, A, 4,514,499 (Noll) 30 April 1985, see column 5, line 27-50 and column 6, lines 1-9.	7,8,53
Y	Dexter et al, " Long Term Bone Marrow Culture", published 1984 by Alan R. Liss, Inc., see page 83.	1
Y	Nature, volume 309, issued 28 June 1984, Dexter, "The message in the medium", pages 746-749, see chart on page 747.	19-22

* Special categories of cited documents:[16]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
02 APRIL 1992	04 MAY 1992
International Searching Authority[1]	Signature of Authorized Officer[20]
ISA/US	Suzanne Ziska, Patent Examiner

Form PCT/ISA/210 (second sheet)(May 1986) ß

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET	
Y	Blood, volume 75, number 11, issued 01 June 1990, Coutinho _et al_, "Effects of Recombinant Human Granulocyte Colony-Stimulating Factor (CSF), Human Granulocyte Macrophage CSF and Gibbon Interleukin -3 on Hematoporeses in Human Long-Term Bone Marrow Culture", pages 2118-2129, see entire article.	1 - 6 , 1 0 - 15,18,23 - 26,33,34,35 - 45,46,47,48,49 ,50,51,54,55
Y	The Canadian Journal of Chemical Engineeing, volume 64, issued August 1986, Adamson _et al_, "Industrial Mammalian Cell Culture", pages 531-539, see entire article.	1 - 6 , 1 0 - 15,18,23 - 26,33,-35 - 45,47,48,50,51 ,54,55,52

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _, because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. ☐ Claim numbers _, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. ☐ Claim numbers _, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

Group I, claims 1-50,51,54,55
Group II, claim 52
Group III, claim 53

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

☐ The additional search fees were accompanied by applicant's protest.

☒ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (supplemental sheet(2))(Rev. 4-90) B

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No.[18]
Y	US, A, 4,963,489 (Naughton et al) 16 October 1990, see entire document.	47
Y	Science, volume 248, issued 15 June 1990, Wilson et al, "Correction of CD18-Deficient lymphocytes by Retrovirus-Medicated Gene Transfer", pages 1413-1416, see page 1415 last column, last paragraph.	9,16,17,27-29,30-32